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Action of the Chaperonin GroEL/ES on a Non-Native Substrate Observed with Single-Molecule FRET

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Abstract

The double-ring-shaped chaperonin GroEL binds a wide range of non-native polypeptides within its central cavity and, together with its cofactor GroES, assists their folding in an ATP-dependent manner. While the conformational cycle of GroEL/ES has been extensively studied, little is known about how the environment in the central cavity affects substrate conformation. Here we use the von Hippel-Lindau tumor suppressor protein (VHL) as a model substrate for studying the action of the GroEL/ES system on a bound polypeptide. Fluorescent labeling of pairs of sites on VHL for FRET allows VHL to be used to explore how GroEL binding and GroEL/ES/nucleotide binding affect substrate conformation. On average, upon binding to GroEL, all pairs of labeling sites experience compaction relative to the unfolded protein while single-molecule FRET distributions show significant heterogeneity. Upon addition of GroES and ATP to close the GroEL cavity, on average further FRET increases occur between the two hydrophobic regions of VHL, accompanied by FRET decreases between the N- and C-termini. This suggests that ATP- and GroES-induced confinement within the GroEL cavity remodels bound polypeptides by causing expansion (or racking) of some regions and compaction of others, most notably, the hydrophobic core. However, single-molecule observations of the specific FRET changes for individual proteins at the moment of ATP/GroES addition show that a large fraction of the population shows the opposite behavior, that is, FRET decreases between the hydrophobic regions and FRET increases for the N- and C-termini. Our time-resolved single-molecule analysis reveals the underlying heterogeneity of the action of GroES/EL on a bound polypeptide substrate which may arise from the random nature of the specific binding to the various identical subunits of GroEL, and may help explain why multiple rounds of binding and hydrolysis are required for some chaperonin substrates.

Keywords

GroEL; VHL; single-molecule fluorescence; FRET; conformational change

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Introduction

Chaperonins are essential for the folding of many proteins *in vivo*, and assist in the folding of newly synthesized proteins in an ATP-dependent manner¹. (For reviews, see^{2–7}.) These double-ring cylindrical assemblies contain a central cavity, where nonnative polypeptides bind and eventually reach the folded state, typically requiring ATP hydrolysis. Chaperonins are classified into two structurally distinct classes. The well-characterized group I chaperonin, GroEL, is found in bacteria and organelles of endosymbiotic origin. GroEL is composed of 14 identical subunits⁸ and its cofactor GroES acts as a lid by binding to GroEL in the presence of ATP⁹. After binding to GroEL, the substrate is usually released and encapsulated in the cavity by GroES and ATP binding. After ATP hydrolysis, both substrate and GroES are released into the solution¹⁰. In contrast, the group II chaperonins have a built-in lid controlled by ATP hydrolysis, with examples such as TRiC/CCT found in eukaryotic cells and the homologous thermosome in archaea^{11, 12}.

The mechanism by which the GroEL-GroES system (herein GroEL/ES) promotes protein folding has been a focus of intense research (for reviews, see^{5, 13}). GroEL/ES can fold a wide spectrum of bacterial and eukaryotic proteins^{14–17}, suggesting it possesses a fundamental ability to conformationally remodel polypeptides to facilitate their folding. One key aspect is the importance of hydrophobic patches in the cavity as binding sites for exposed hydrophobic regions of unfolded substrates; these regions become hydrophilic upon ATP/GroES binding and substrate encapsulation¹⁸. Several previous single-molecule studies examined GroEL/ES binding interactions with various substrates of GroEL^{16, 19–21}. In this study, we explore the interactions between GroEL/ES and the human von Hippel-Lindau (VHL) tumor suppressor protein, used as a model poly(peptide) substrate of GroEL/ES containing hydrophobic and hydrophilic regions. Ensemble and single-molecule FRET studies were used to monitor the substrate conformational changes that occur during binding of VHL to GroEL and, furthermore, the time-dependent FRET changes which occur upon the assembly of the GroEL/ES complex with ATP.

Results and discussion

VHL as a model substrate for GroEL/GroES

VHL normally interacts with the chaperones TRiC and Hsp70 in eukaryotic cells in order to form its active folded complex with two additional accessory proteins Elongin B and C^{22–24, 24}. These interactions are mediated by two hydrophobic regions on VHL, named the Box1 (B1) and Box2 (B2) regions, which are necessary and essential for chaperonin binding^{23, 25}. These regions are distant in the sequence but are located in adjacent regions in folded VHL (Figure 1ab)²⁶. VHL can also interact with the prokaryotic chaperonin GroEL and can reach its properly folded state in the presence of Elongin B/C via ATP-driven interaction with GroEL/ES (see Fig. S1, Supplemental Material). However, since the fully folded state of VHL is only formed in the presence of Elongin B/C, study of the complete folding process of VHL would require the assembly of multiple components and is not of primary interest here. Rather, we view VHL as a model poly(peptide) containing two hydrophobic regions B1 and B2 known to drive binding to chaperonins²⁵, and concentrate on how the conformation of VHL changes during VHL-GroEL binding and during the formation of the VHL-GroEL/ES complex in the presence of nucleotides. In this way, specific information can be obtained about the conformational changes produced by the GroEL/ES system on a non-native substrate. This study thus provides information regarding the nonspecific, ubiquitous ability of the chaperonin to assist many substrates.

The structure of VHL after binding to GroEL is more compact

In order to characterize the conformational changes of VHL, we applied the well-known Förster Resonance Energy Transfer (FRET) method^{27, 28} to probe changes in pairs of amino acids of VHL during interactions with GroEL, first in ensemble measurements and subsequently in the single-molecule regime. The fluorophores Alexa488 (AX488) and Texas Red (TR) were chosen as a FRET pair to label double-cysteine variants of VHL. This pair of dyes has a relatively small R_0 value (~ 46.5 Å)²⁹, which is necessary for monitoring a small protein like VHL. Six different double Cys variants were prepared and labeled with both AX488 and TR maleimide. The labeling positions and the β -carbon distances between each amino acid pair for VHL in its native complex with ElonginBC and the sequence separations are shown in Fig. 1ab. Importantly, the N and C positions report on the relatively polar termini of the protein, whereas the B1 and B2 positions report on the two hydrophobic core regions²⁵.

Fig. 1c shows the relative bulk solution FRET efficiencies (expressed as a proximity ratio $F = I_A / (I_A + I_D)$) for six different variants of VHL in three different conditions; 6M GdnHCl, native buffer, and bound to GroEL in native buffer. As described in Materials and Methods, any unbound VHL that could lower the FRET efficiency was removed using a gel filtration column before each measurement. In the unfolding conditions of 6M GdnHCl, FRET efficiencies for all variants were the lowest compared to all the other conditions. As may be expected, the FRET value was roughly inversely proportional to the sequence separation between the labeling sites in the polypeptide chain (data not shown)^{30, 31}. For example, the F value for the B1-B2 variant (36 aa separation) in 6M GdnHCl was highest (~ 0.50), while that of the N-C variant (122 aa separation) had the lowest value (~ 0.12) in the unfolded state. For VHL in native buffer (light gray columns in Fig 1c), the degree of FRET was larger than for VHL in 6M GdnHCl for all six variants, suggestive of the collapse observed by others³¹. Finally, the FRET proximity ratio for all VHL variants was significantly increased when VHL was complexed with GroEL (dark gray columns in Fig. 1c). The N-B1 variant showed more than ~ 10 Å contraction after binding to GroEL (roughly extracted from F value increase from 0.39 to 0.72, see Materials and Methods for controls and equations used in distance estimates). These results suggest that simple binding of VHL to the chaperonin passively induces significant conformational changes in VHL, specifically compaction with respect to a random coil extended conformation.

Single-molecule FRET reveals position-dependent conformational changes in the substrate upon lid closure

Next, we measured FRET efficiencies for six VHL double variants with GroEL/ES at the single-molecule level. In order to immobilize individual molecules for long-term observation, we prepared biotin-labeled GroEL (C473Bio-EL) and bound it to a glass coverslip by biotin-Neutravidin interactions. A carefully prepared PEG-coated glass coverslip with a small amount of biotin bound to the surface was used to avoid any non-specific GroEL binding to the surface (see Fig. 2a). Several control experiments demonstrated that we observed the functional GroEL-VHL complex, rather than non-specifically bound forms of VHL protein on the surface. First, in the absence of Neutravidin, no Cy3-labeled C473Bio-EL test molecules, no fluorophore-labeled VHL, and no biotin-labeled GroEL in complex with VHL were observed stuck to the PEG-coated surface. Both the amount of Neutravidin and the incubation time for biotin-Neutravidin interaction were critical to observe any fluorescence from the VHL, and therefore non-specific binding of VHL to the surface was not occurring. Secondly, we measured the single-molecule emission polarization of the VHL-GroEL complex after immobilization on the glass coverslip (see Fig. 2b). Since we used a biotin molecule with a long linker between the biotin and the maleimide to label C473EL (Biotin-PEG-maleimide with MW 3810), this complex should

be free to rotate after immobilization if there is no strong protein-surface interaction. AX488 dye molecules spun on an unpassivated glass coverslip showed a broad, heterogeneous polarization (P) distribution due to non-specific binding, while both AX488- and the TR-labeled VHL(N terminal labeled)-GroEL complexes bound to the prepared surface had narrow P distributions centered at P=0. All the other single mutants (B1, B2, and C) with either of the two different fluorophores (total 6 samples) also showed similar narrow distributions, demonstrating that GroEL-VHL complexes are free to rotate without any specific interaction with the surface on the 0.1 sec time scale of our measurements. (For fluorescence anisotropy measurements on the scale of the excited state lifetime, see Materials and Methods.) Single-molecule FRET measurements of VHL only in the absence of GroEL were attempted by immobilizing VHL in 1.5% agarose gel. However, due to the additional heating/cooling process, the conformation of VHL in agarose gel was disturbed. In fact, we observed increased average FRET in these perturbed cases (data not shown).

Fig. 3 shows the time-averaged single-molecule FRET efficiency distributions for four of the double variants denoted by the two labeling positions (N-B1, B1-B2, B2-C, and N-C). The upper row shows the distributions for unfolded VHL complexed with GroEL only, the lid-open state. The dashed lines locate the average FRET values for each case. In the lower row of Fig. 3, the lid-closed complex has been formed by incubation of labeled VHL with GroEL, GroES, and ATP/AlFx^{21, 32, 33}, a transition state mimic of ATP hydrolysis. It has been shown that addition of ATP or ADP with AlFx stopped the ATPase cycle of GroEL, producing a symmetric complex (bullet form) of GroEL/ES. In this case, both GroEL cavities are closed by GroES. For two variants, N-B1 and B2-C, the mean of the distributions did not shift appreciably (as was the case for two other variants, N-B2 and B1-C, not shown), although the distribution showed a greater width extending to lower FRET values for the N-B1 variant. However, significant shifts appeared for the B1-B2 and N-C variants. While the degree of FRET shifted to higher values for the B1-B2 case suggesting a decrease in the B1-B2 distance, the distribution shifted to lower values for the N-C variant suggesting an increase in the N-C distance. This suggests that confinement of a non-native polypeptide within the GroEL/ES cavity leads to compaction of the hydrophobic core but expansion of the overall conformation of the polypeptide.

One of the advantages of single-molecule measurements is that conformational heterogeneity can be estimated by the width of the distribution²⁸. Importantly, the distributions for all cases were much wider than can be explained by experimental noise arising from the number of photons detected. The standard deviation of the shot noise distribution, calculated from the method in Gopich et al.³⁴ (see Materials and Methods) was about 0.005 for all cases in Fig. 3, which is ~20 times smaller than the observed widths. Therefore, it is likely that a large degree of conformational heterogeneity is present in VHL bound to GroEL in the open and closed states of the chaperonin. All six variants of lid open state (upper row in Fig. 3) showed similarly shaped efficiency distributions with similar widths (standard deviation of about 0.09, i.e. 3.0 Å in distance, on average). In the case of the N-C variant and the N-B1 variant, a further increase in the distribution width could be observed in the lid-closed state

Dynamic single-molecule measurements upon ATP addition reveal the stochastic or probabilistic nature of GroEL/ES substrate interactions

Dynamic (time-dependent) single-molecule FRET measurements were performed to follow the interaction between VHL and GroEL upon ATP and GroES addition in real time. Only the B1-B2 and N-C variants were considered here, because these two pairs sample the two extremes: the overall protein structure (N-C), and the highly hydrophobic regions providing the likely chaperonin binding region (B1-B2). Before interpreting any ATP dependent

changes, the effect of photobleaching of the fluorophores must be addressed. As usual, photobleaching is determined on the single-molecule level by the disappearance of a single-molecule spot; however, here we must distinguish between photobleaching and spot disappearance due to release of the labeled VHL from the surface-attached chaperonin. First, we measured the survival distribution of AX488 bound to the actual protein by recording continuous images of singly-labeled AX488-VHL bound to surface-attached GroEL with 0.1 sec integration time and found a characteristic $\tau_{\text{bleach}} \sim 4.1$ sec (Fig. 4a). To follow conformational changes induced by ATP addition over longer times before fluorophore photobleaching, time-lapse imaging was then implemented using a 0.1 sec exposure and a 0.9 sec dark interval. This had the effect of extending the photobleaching time to 41 sec (41 cycles of time-lapse), much longer than the estimated ATP hydrolysis time of GroEL (~ 15 sec¹⁰). GroEL has two distinct rings, and the GroEL capped with GroES is called the *cis* ring, while the non-capped ring is called the *trans* ring. As has been shown, upon ATP hydrolysis to ADP and binding of an additional ATP to the *trans* ring, the GroES-GroEL complex is destabilized, leading to both GroES and substrate release from the *cis* GroEL¹⁰. Following substrate release, the fluorescent spot would then disappear as the fluorescent substrate diffuses away from the surface-attached chaperonin. Using this experimental strategy, the fluorescence from VHL (complexed to GroEL) on the surface was monitored for $t = 15$ sec (15 cycles of time-lapse) prior to nucleotide addition, and then the subsequent effects of ATP-induced interaction changes were investigated. This regimen allowed us to have enough data points both before and after ATP addition to observe time-dependent changes with 1 sec time resolution.

When ATP was added to the AX488-VHL-GroEL complex on the surface, roughly 10% of the AX488-VHL single-molecule spots quickly disappeared within 5 sec, compared to the case with no ATP addition. A further decrease ($\sim 40\%$) in the number of bound single molecules was observed when GroES was present in the solution before ATP addition (Fig. 4a inset), and this was also observed with all of the other mutants. The fast disappearance of a fraction of the single-molecule spots upon ATP addition was likely due to release of VHL into the solution during *cis* or *trans* ATP/GroES complex formation^{10, 35}, rather than encapsulation into the *cis* cavity. A similar partial “loss” of substrate during encapsulation was also reported by Weissman et al. where they observed that only 30–40% of rhodanese substrates were encapsulated after lid closure³⁶. Under time-lapse conditions (where the effective photobleaching/disappearance time is 41 sec on average), the addition of ATP in the presence of GroES caused the disappearance time constant to decrease to 30 sec (inset of Fig. 4a), which reflects the ATP hydrolysis and complex disassembly time scale.

The fraction of single-molecule spots which did not disappear after ATP/ES addition remained associated with the chaperonin and likely encapsulated, and the FRET signal for these complexes was analyzed. Fig. 4b shows example traces for a B1-B2 variant (left side) and a N-C variant (right side). Note that in each case, the donor and acceptor signals (upper panels) and the FRET signal (lower panels) were monitored before and after the moment of ATP addition (solid arrow). For the B1-B2 case (left side), the FRET value increased upon closure of the cavity, followed by photobleaching at 40 sec total elapsed time. This molecule is therefore an example of the average behavior of Fig. 3: closure of the cavity induced a compaction between the B1 and B2 hydrophobic regions. For the N-C case (right side) the FRET value decreased upon ATP-induced closure of the cavity, followed by photobleaching at 37 sec total elapsed time. This molecule is also an example of the average behavior of Fig. 3: closure of the cavity induced an expansion between the N and C hydrophobic regions.

Importantly, because the actual FRET signal is followed for each single molecule before and after ATP addition, the conformation of VHL simply bound to GroEL can be directly correlated with its conformation after complex formation. Fig. 5ab shows this by displaying

the distance (extracted from properly corrected efficiency values) between two probes before and after ATP addition in a scatter plot for many single-molecule traces like Fig. 4b (101 molecules for B1-B2, 80 molecules for N-C mutant were observed). The data fall into two regions separated by the line representing no distance change. Due to the large fluctuation of the FRET signals, we applied Welch's t-test to each molecule and present only those transitions where the difference in mean efficiency before/after ATP addition was significant using a 95% confidence interval; this criterion selected about 40% of all the molecules studied. As expected from the average efficiency changes in Fig. 3, 84% of the B1-B2 molecules showed a distance-decrease transition, and 69% of the N-C molecules showed the opposite change, a distance increase. Of note, a minority of the molecules showed the opposite behavior. For B1-B2, ATP addition was followed by a distance *increase* for 16% of the molecules (or drop in FRET), and for N-C, ATP addition was followed by a distance *decrease* for 31% of the molecules (or increase in FRET). Therefore, the structural changes upon ATP binding appear to be heterogeneous and probabilistic.

We also considered the possibility that the conformation of the VHL still bound to the GroEL/ES complex might change with time after the moment of complex assembly induced by ATP addition (data not shown). Taking into account the noise in the FRET signals, no evidence was found for further significant conformational changes after cavity closure.

A picture of the action of GroEL/ES on a bound polypeptide

Despite intense study, it is still unclear in detail how GroEL affects substrate conformation to facilitate folding. Several previous studies examined the conformational changes—either compaction or expansion of GroEL substrates before and after GroES lid closure at the bulk level^{37–39}. Our work provides complementary information on how GroEL binding affects the conformation of a bound polypeptide both at the bulk and single-molecule level, as summarized in Fig. 5c. Upon binding to GroEL, the protein becomes more compact relative to its conformation in denaturant, with a shortening of distances between all regions in the protein. For example, the estimated distance between N-C termini is about 45 Å. Following substrate encapsulation upon ATP and GroES addition, the hydrophobic core becomes more compact on average, whereas the N-C distance increases on average. This is not due simply to folding, since VHL can only reach the final native state following release from the cavity and binding to its oligomeric partners ElonginBC.

This work finds significant heterogeneity, both in the average FRET distributions (Fig. 3) and in the time-dependent changes in FRET induced by EL/ES cavity assembly induced by ATP (Figs. 4b and 5ab). Recent single-molecule studies by both Hillger et al.¹⁹ and Sharma et al.³¹ also described heterogeneous populations of rhodanese and mutant MBP upon binding to GroEL as well as further conformational changes upon ATP addition. Both rhodanese and MBP are present with GroEL/ES in prokaryotic cells, while VHL did not co-evolve with the bacterial proteostasis network. This might explain some fraction of the heterogeneity we observe, but it is unlikely to explain all of the heterogeneity. For the case of VHL, the majority behavior shows an ATP- and GroES induced compaction for B1-B2 and expansion for N-C, but there is a significant minority of the single molecules with the opposite behavior. These results may be rationalized with a simple physical picture. In the open, unliganded state, GroEL exposes hydrophobic binding sites in the apical domains of each subunit of the ring⁸. It is reasonable that the two hydrophobic regions of VHL (B1 and B2) take the lead in determining the interaction with GroEL, as with TRiC²⁵. Each of the seven subunits in one GroEL ring has identical hydrophobic patches, each of which can attract exposed B1 or B2 for binding. One then might guess that the B1 and B2 regions could bind to adjacent GroEL subunits, or to subunits one or two positions apart. For example, the distance between two adjacent binding sites calculated from the crystal structure is about 23 Å. Referring to the FRET efficiency distribution, it is likely that most

of the B1-B2 cases bind to the subunits two monomers apart. But this is only the average case - for some molecules B1 and B2 would be brought closer together as the apical domains move, but for other single molecules, especially those whose B1-B2 positions are too closely located at the starting point after binding to GroEL, B1 and B2 would be moved further apart from their starting separation, showing expansion. On the other hand, the N- and C- termini, not being hydrophobic, would not be expected to bind strongly to GroEL. The relative conformational expansion between these two locations may be a secondary result of the changes imposed by B1 and B2 binding or may result from the changed, more hydrophilic environment of the closed GroEL/GroES cavity. It is also possible that solvent effects account for the conformational rearrangement observed upon GroES binding⁴⁰⁻⁴².

Clearly, more work will be required to clarify the basis of the observed substrate remodeling and its probabilistic behavior from molecule to molecule. Taken together, these results clarify the idea that the average action of GroEL/ES on exposed hydrophobic regions is to move them closer together, an effect that would be expected to ultimately promote folding. For those molecules which did not bind in a favorable orientation, the action of GroEL/ES in promoting folding is thwarted, and therefore, additional rounds of binding and hydrolysis would be expected to stochastically sample the other possibilities available. This stochastic nature has been observed directly (for different molecules) in this single-molecule experiment. In principle, the stochastic behavior may reflect the ability of GroEL/ES to assist in exploration of a larger range of conformational space for the substrate in the attempt to fold non-evolved substrates.

Materials and methods

Protein Purification

The purification of VHL, GroEL, GroES, VHL fluorescent labeling, and GroEL-biotin labeling are described in the Supplementary Material.

Bulk fluorescence measurements

The steady-state anisotropy measurements were performed using a Florolog-3 fluorimeter (Jobin-Yvon ISA Spex, Inc.), while fluorescence measurements utilized a FluoroMax-2 fluorimeter (Jobin-Yvon ISA Spex, Inc.). The fluorescence anisotropy (r) was calculated by $r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH})$, where $G = I_{HV} / I_{HH}$. Alexa Fluor 488 (AX488) was excited at 490 nm, while 590 nm was used for pumping Texas Red (TR) acceptor molecules. The bulk FRET proximity ratio was determined by recording fluorescence from double-labeled proteins using 490 nm excitation, and computing $F = I_a / (I_a + I_d)$, where I_a and I_d are fluorescence intensity of acceptor and donor, respectively. To prepare samples for fluorescence measurements, denatured, labeled VHL (10 μ M) was diluted into 100 nM of either wild type GroEL or Cys473-biotin GroEL (C473Bio-EL) solution, and incubated for 20 min. at 30°C in buffer A. In case of the GroEL-VHL complex with ATP/ AlF_x , 30 mM NaF, 5 mM $AlNO_3$, and 2 mM ATP were added after complex formation, and then incubated further for 30 min. Then GroES was added and incubated for 10 min. to form a lid-closed complex (two GroES molecules bound at the end of each GroEL ring). To remove any unbound VHL, the solution was always filtered with a Micro Bio-Spin P30 column (Bio-Rad) after complex formation. Because exact concentrations varied, F is reported as a measure of FRET in the bulk measurements rather than true FRET efficiency values (see below).

Sample preparation for single-molecule measurements

Protein solutions were prepared as described above. A PEG coated coverslip with a small amount of biotin PEG was prepared as described for protein immobilization⁴³. This

coverslip was assembled in a flow cell chamber (RC-24, Warner Instruments), and then incubated with Neutravidin (0.1 mg/mL from Pierce). After removing any unbound Neutravidin by flowing buffer A, Cys473Bio-EL and VHL complex (or Cys473Bio-EL, VHL and GroES complex with ATP/AlF_x, lid-closed complex) was added to the chamber (~300 pM final). To reduce background coming from unbound EL-VHL complexes, the chamber was washed with buffer A before imaging after 5 min. incubation. For the lid-closed complex, the same amount of salt and ATP were added to the buffer A to maintain equilibrium. Lastly, dynamic (time-dependent) FRET measurements upon ATP addition were performed by dropping 2 mM ATP (final concentration) to the chamber.

Single-molecule fluorescence imaging

Both single-molecule FRET and polarization measurements were performed using an epifluorescence configuration with an Olympus IX71 inverted microscope. When backgrounds are carefully controlled and all emitters are located on a surface, epifluorescence easily produces single-molecule images⁴⁴. To excite AX488 molecules, a Novalux Protera frequency-doubled semiconductor diode laser with a wavelength of 488 nm was used (0.1 kW/cm²). Fluorescence was collected with a 100X oil immersion objective (NA 1.35) and filtered through a 495DRLP dichroic (XF2026, Omega Optical) and 500LP long pass filter (Omega Optical). For pumping TR, a yellow HeNe laser (594 nm, Coherent) was used as an excitation source. A 600DRLP dichroic (Omega Optical) and a 620 LP long pass filter (Omega Optical) were used to separate scattered excitation light from the fluorescence. Images were recorded with an EMCCD camera (Andor Ixon, DV887ECS-BV). To capture the image of both donor (AX488) and acceptor (TR) molecules simultaneously for FRET measurements, an image splitting device (Optosplit II, Cairn Research) was placed before the camera. A 560 DRLP dichroic (Omega Optical) was used to separate two emission colors, and two extra bandpass filters (HQ525/50M from Chroma and 610/70M from Omega Optical) were used to avoid any crosstalk. Images were continuously recorded with a 0.1 sec integration time. In order to exclude any donor-only molecules, we first excited TR molecules with the 594 nm beam to locate TR molecules (less than 0.2 sec). Then the excitation beam was switched to 488 nm to excite AX488 molecules, and images of both the donor emission and the acceptor emission were recorded. In each measurement, we only analyzed molecules that had both the TR acceptor and the AX488 donor present. Further information regarding calculation of the single-molecule FRET efficiency, R_0 , and inter-fluorophore distance is described below.

Single-molecule polarization measurements

The same single-molecule imaging setup was used for single-molecule fluorescence polarization measurements, except that circularly polarized excitation was generated by a quarter-wave plate and a 50/50 polarizing beam splitter cube was used in the image splitter instead of a dichroic to separate the emission into two differently polarized beams^{45, 46, 46}. The polarization factor was calculated using $P = (I_x - f I_y) / (I_x + f I_y)$, where f is a weighting factor to compensate for different detection efficiency⁴⁷. The factor f was determined using singly-labeled VHL in 2% agarose gel with 6M GdnHCl solution. Images were continuously recorded with a 0.1 s integration time. AX 488 single molecules spun directly on a glass coverslip showed a broad, heterogeneous distribution of P values (Fig. 2b, left) due to non-specific binding. However, both AX488 and TR labeled VHL(N)-GroEL complexes immobilized on the PEG-coated glass coverslip had narrow distributions centered at $P=0$ (Fig. 2b, middle and right, respectively). All the other single mutants (B1, B2, and C) with two the different fluorophores (total 6 samples) also showed similar distributions as shown in Figure 2b, demonstrating that the fluorophores on VHL making the GroEL-VHL complexes visible are free to rotate without any specific interaction with the surface in this time scale.

Calculation of FRET efficiency and R_0

Single-molecule FRET efficiency values E were calculated by the following equation ⁴⁶

$$E = \frac{I_A^{Corr}}{I_D^{Corr} + I_A^{Corr}} = \frac{1}{1 + \frac{\varphi_A}{\varphi_D} \frac{D}{\left(\frac{I_A}{I_D} - L\right)}}$$

where I_A and I_D are the measured intensities, and D ($D = 1.009$) is the ratio between the different detection efficiencies for donor and acceptor caused by filters and dichroics. L ($L = 0$ here) is the donor leakage into the acceptor channel, measured from donor-only samples. φ_D (0.42) and φ_A (0.39) are the measured fluorescence quantum yield of donor and acceptor. For our determinations of quantum yield for AX488-VHL and TR-VHL with GroEL, we used fluorescein in 0.1 M NaOH ($\varphi = 0.95$) ⁴⁸, and sulforhodamine 101 in methanol ($\varphi = 0.90$) ⁴⁹ as references.

To convert FRET efficiency to distance, R_0 was calculated from the equation,

$$R_0 = 9.78 \times 10^3 (n^{-4} \kappa^2 \varphi_D J)^{1/6}$$

using $J = 1.84 \times 10^{-13} \text{ M}^{-1} \text{ cm}^{-3}$ from measured spectral overlap between AX488 emission and TR absorption. A refractive index of 1.345 (10% glycerol) was used for the buffer solution, and R_0 was calculated to be 48.3 Å. For this R_0 calculation, we assumed that the orientational factor κ^2 approached the orientationally averaged value of 2/3. The distance R was then calculated from the equation,

$$R = \left(\frac{1 - E}{E} \right)^{1/6} R_0$$

where E is the energy transfer efficiency.

To confirm that the rate of the dye's rotation was faster than the lifetime of the dye, both ensemble-averaged anisotropy and time-resolved anisotropy decay measurements were performed. Time resolved anisotropy decay measurements were performed using EasyLife II from PTI. As an excitation source, a 470 nm pulsed LED (FWHM = 1.7 ns) was used to excite the AX 488 molecule. Two polarizers were placed before and after sample, and emission from the sample was collected with a GG495 long pass filter. To calculate G factor ($G = I_{HV}/I_{HH}$), both I_{HH} and I_{HV} , as well as I_{VH} and I_{VV} were recorded. A diluted colloidal silica solution (Ludox from Sigma-Aldrich) was used to record an Instrument Response Function (IRF). The anisotropy decay was fitted by FeliX GX software from PTI.

The ensemble-averaged anisotropy values of VHL-chaperonin complex were similar for all mutants, suggesting that the different positions of the probe were not important, and implying that the probe reports the whole protein motion, rather than being affected by particular local conformation. The anisotropy value of AX488 labeled VHL was about 0.34 for the GroEL-VHL complex, while it dropped to ~0.27 after the lid closure, probably due to rapid motion of labeled VHL after release of VHL from the apical domain. Still, VHL was rotationally constrained within the cavity and showed higher anisotropy values than for free VHL in aqueous buffer ($r \sim 0.2$) ^{10, 50}.

Next, we compared the anisotropy decay of free AX488 in buffer with the AX488 labeled VHL-GroEL complex. Due to experimental limitations arising from the IRF (FWHM = 1.7 ns), we could not fit the fast decay. While the free AX488 decay immediately dropped to ~0, the AX477VHL-EL anisotropy stayed at a value = ~0.3, probably due to global protein motion (~1 μ s, reported with pyrene-labeled GroEL⁵¹). As a control, the anisotropy decay of AX488 in 50% glycerol was measured, and the rotational lifetime of AX488 was ~ 1.5 ns. Furthermore, careful study with the same FRET pair by Slaughter et al. demonstrated that at least the rotational lifetime of the donor AX488 was clearly faster than the fluorescence lifetime under various conditions²⁹. Therefore, we assumed that AX488 had a large rotational freedom, and therefore $\kappa^2 = 2/3$ was an appropriate approximation. Although the anisotropy decay of TR labeled VHL with GroEL was not measured, the rotational freedom of AX488 was sufficient for this approximation^{29, 52}.

The variance of the extracted FRET efficiency values arising from shot noise is calculated by the following equation³⁴

$$\sigma_p^2(E_m, N, n) < \frac{\langle E_m \rangle (1 - \langle E_m \rangle)}{\langle N \rangle n}$$

where E_m is the mean energy transfer efficiency, N is the mean of the number of photons in a given time, and n is the number of measurements (number of frames that each molecule lived). The number of photons was calculated considering the loss of fluorescence due to the microscope geometry, the quantum efficiency, conversion factor and amplification factor of the EMCCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VHL	von Hippel-Lindau tumor suppressor protein
FRET	Fluorescence (Förster) Resonant Energy Transfer
AX488	Alexa-Fluor 488
TR	Texas Red
C473Bio-EL	biotin-labeled Cys473 variant GroEL
GdnHCl	Guanidinium hydrochloride
PEG	poly(ethylene glycol)

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Figure 1.

(a) The crystal structure of VHL in the VBC complex and the locations of Cys mutations and the inter-residue distances in this study. Hydrophobic binding sites are highlighted in red (Box1 or B1) and blue (Box2 or B2). (b) The table shows the sequence separation in number of amino acids and the distance between two positions in the VBC complex for the various double-Cys variants. (c) Bulk FRET efficiencies estimated by the proximity ratio F for 6 VHL variants in 6M GdnHCl, in native buffer, and with GroEL. The double-labeling efficiency was ~95%, as HPLC purification allowed separation of the doubly-labeled fraction from singly-labeled and unlabeled. For the native buffer case, denatured VHL in 6M GdnHCl was diluted into buffer A (50 mM HEPES-KOH (pH 7.4), 50 mM KCl, 10 mM MgCl₂, 10% glycerol, 1mM DTT), with a 1:100 volume ratio (100 nM final concentration). To confirm that VHL was not aggregated in this condition, we separately used donor-only labeled and acceptor-only labeled VHL, diluted into the native buffer with the same final concentration, and found no significant FRET, indicating that VHL is not aggregated in this condition. In the case of VHL with GroEL, we removed any unbound VHL by treating the solution with a spin column after incubation. This causes a concentration variation of each sample, and therefore the proximity ratio is reported as a measure of relative FRET efficiency.

Figure 2.

(a) PEG-coated glass coverslips were prepared as described by Joo, et al.⁴³ (Bottom) A specific binding test was performed using biotin- and Cy3-labeled GroEL (C473Bio-EL). In the presence of Neutravidin (left panel), Cy3-labeled C473Bio-EL could be viewed on the surface, producing more than ~30 fluorescence spots in the viewing region (~10×10 μm). On the other hand (right panel), as a negative control, after washing any unbound C473Bio-EL, only 1–2 fluorescence spots could be observed in the absence of Neutravidin. (b) Single-molecule polarization histograms of the AX488 dye spun on a glass coverslip (left), AX488 labeled VHL (N)-GroEL (middle), TR labeled VHL (N)-GroEL (right). For the latter two cases, single VHL-GroEL complexes were immobilized on the PEG-coated glass coverslip using the biotin-Neutravidin linkage. The narrow distribution centered at $P=0$ for the VHL-GroEL complexes implies that the GroEL-VHL complexes are free to rotate without any specific interaction with the surface on the 100 ms time scale.

Figure 3.

Single-molecule FRET efficiency distributions measured with individual VHL-GroEL molecules. After preparation of the biotin-PEG coated surface, complexes were prepared by diluting unfolded VHL into buffer A with C473Biotin-EL for 20 min, incubated on the surface for 5 min, and the surface was then washed before imaging. To produce the lid closed state, the VHL-C473Biotin-EL complex was further incubated with ATP/AlFx for 30 min, and then with GroES for 10 min. Single molecules were imaged with 488 nm pumping in two color detection channels to record both the emission from the donor and from the acceptor simultaneously. Illumination was performed in the time-lapse mode for 100 ms followed by a dark period of 10 s; under these conditions single molecules survived for time periods on the order of 10 s total elapsed time (see Fig. 4). Four VHL variants labeled at two positions (N-B1, B1-B2, B2-C and N-C) are shown in this figure. The upper row shows the case for the lid-open state, i.e., GroEL-VHL complexes alone. Dotted lines in each graph indicate the average FRET efficiency for the lid-open state. The lower row shows the case for the lid-closed state produced by the addition of GroES and ATP/AlFx.

Figure 4.

(a) The number of surviving molecules distribution for single molecules of AX488 VHL (N) with GroEL. Continuous illumination with 0.1 sec integration time was used to measure the number of molecules remaining after specific illumination times to assess photobleaching, while time-lapse imaging (0.1 sec exposure with 0.9 sec dark interval, inset) was used for longer time observations. The continuous illumination distribution was fit with a single exponential, with the time constant $\tau = 4.1$ sec. The inset shows the number of surviving molecules under time-lapse conditions with abscissa showing in real laboratory elapsed time with ATP addition in the presence of GroES. ATP was added after 15 cycles of time-lapse (dotted line), which corresponded to $t = 1.5$ sec of actual illumination. The number of molecules visible on the surface significantly dropped right after ATP addition, due to the disappearance of some VHL molecules caused by release of some VHL molecules into the solution. (b) Examples of time-lapse single-molecule fluorescence traces of donor/acceptor emission and calculated FRET efficiency values for GroEL-bound VHL before/after ATP addition with GroES present in the solution. This is a complex where VHL did not immediately dissociate after ATP addition. Arrows indicate when ATP was added ($t = 15$ sec). The example on the left (a B1-B2 variant) showed increased FRET after ATP addition and photobleaching at 40 sec, whereas FRET decreased in the N-C example on the right with photobleaching at 37 sec.

Figure 5.

Characterization of individual FRET transitions of (a) single B1-B2 and (b) single N-C molecules upon ATP addition. The calculated distances extracted from true FRET efficiency E before and after ATP addition were used as x- and y- coordinates. Only transitions with significant changes before/after ATP addition are shown. The solid lines represent no change after ATP addition. In the case of the B1-B2 variant, 84% of molecules showed compaction (distance-decrease transition) after ATP addition, while 69% of N-C variant molecules showed expansion (distance-increase transition). (c) Summary of the structural changes of VHL upon binding to GroEL followed by cavity closure (only one ring shown). First, unfolded VHL shows compaction upon binding to GroEL. Further structural changes could be observed after ATP-induced lid closure, but these were quite heterogeneous with net average compaction for B1-B2 and net expansion for N-C.