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Concerted release of substrate domains from GroEL by ATP is demonstrated with FRET

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Summary

The chaperonin GroEL assists protein folding by undergoing ATP-induced conformational changes that are concerted within each of its two back-to-back stacked rings. Here we examined whether concerted allosteric switching gives rise to all-or-none release and folding of domains in a chimeric fluorescent protein substrate, CyPet-YPet. Using this substrate, it was possible to determine the folding yield of each domain from its intrinsic fluorescence and that of the entire chimera by measuring FRET between the two domains. Hence, it was possible to determine whether release of one domain is accompanied by release of the other domain (concerted mechanism) or if their release is not coupled. Our results show that the chimera's release tends to be concerted when folding is assisted by a wild-type GroEL variant but not when assisted by the F44W/D155A mutant that undergoes a sequential allosteric switch. A connection between the allosteric mechanism of this molecular machine and its biological function in assisting folding is thus established.

Introduction

Oligomeric ring-shaped proteins that undergo ATP binding and hydrolysis-induced conformational changes play a vital role in many biological processes such as protein folding and degradation[1] and unwinding of duplex nucleic acids.[2] These conformational changes may be concerted as in the case of the chaperonin GroEL,[3] sequential as found for the chaperonin CCT[4] or probabilistic as recently proposed for ClpX.[5] The relationship between these different mechanisms of nucleotide-induced conformational changes and the biological function they facilitate is generally not known. Here, we address this issue with regard to the *Escherichia coli* chaperonin GroEL that assists protein folding by undergoing ATP-induced allosteric transitions between protein substrate binding and release states (for reviews see, for example, refs. 3, 6-9). GroEL consists of two homo-heptameric rings, stacked back-to-back, with a cavity at each end[10] in which protein substrate folding can take place in isolation from bulk solution. It has a heptameric ring-shaped cofactor, GroES, which is essential for the assisted folding of stringent substrates under non-permissive conditions.[11] GroEL undergoes allosteric transitions that are induced by ATP binding that occurs with positive intra-ring cooperativity and negative inter-ring cooperativity.[12,13] Experimental data (refs. 14, [15]; G. Curien, J. Grason and G. Lorimer, Department of Chemistry and Biochemistry, University of Maryland

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at College Park, personal communication) and computer simulations[16] have indicated that the intra-ring allosteric transitions of GroEL are concerted in accordance with the Monod-Wyman-Changeux representation[17] as proposed by the nested model.[12] The impact of a concerted allosteric mechanism on the function of GroEL has been unclear.

The work described here was undertaken in order to test the hypothesis that the concerted allosteric transitions of GroEL facilitate the release of the different parts (domains) of a bound protein substrate in an all-or-none fashion. A powerful tool that is available for investigating this question is a wild-type variant of GroEL (F44W18) with the D155A substitution that converts its ATP-induced intra-ring allosteric transitions from concerted to sequential.[19] In other words, this substitution converts the $\mathbf{t}_7 \rightarrow \mathbf{r}_7$ allosteric transition of a wild-type ring into a sequential allosteric transition such as $\mathbf{t}_7 \rightarrow \mathbf{t}_4\mathbf{r}_3 \rightarrow \mathbf{r}_7$ or $\mathbf{t}_7 \rightarrow \mathbf{t}_5\mathbf{r}_2 \rightarrow \mathbf{r}_7$ (**t** and **r** stand for the respective conformations of a subunit in the low (**T**) and high (**R**) affinity states of a ring for ATP and $\mathbf{t}_n\mathbf{r}_{7-n}$ stands for a ring with n adjacent subunits in the **t** conformation and the others in the **r** conformation). In previous work,[20] we fused a stringent (i.e. GroES-dependent) substrate, rhodanese, to the non-stringent substrates mouse dihydrofolate reductase (mDHFR) or enhanced green fluorescent protein (EGFP) and showed that folding of the non-stringent part of the chimeras occurs in a step-wise fashion, with respect to ATP concentration, when folding is assisted by the F44W/D155A mutant but not when assisted by the wild-type variant. In these experiments, only the non-stringent component of the chimeras was able to fold to the native state while the stringent part remained bound and unfolded.[20] In the experiments described here, we employed a chimera comprising the cyan and yellow fluorescent proteins, CyPet and YPet,[21] which are both non-stringent substrates. The folding yield of each domain could be determined separately by measuring its respective intrinsic fluorescence and that of the chimera (with both domains folded) by measuring the Förster resonance energy transfer (FRET) between the two domains. Hence, by using this chimera as a substrate, it was possible to determine whether assisted folding by GroEL (either the wild-type variant or the F44W/D155A mutant) of one domain tends to be accompanied by folding of the other domain (i.e. a 'concerted' mechanism) or whether release and folding of the two domains is independent. We show that a concerted ATP-induced allosteric switch in GroEL results in a more concerted release of substrate domains, thereby linking the allosteric properties of this machine with its folding function.

Results

Acid-denatured chimera was added to F44W or F44W/D155A GroEL and refolding was then initiated by adding different concentrations of ATP. The reactions were allowed to continue until the maximal folding yield was reached at each ATP concentration and fluorescence emission spectra following excitation at 430 and 480 nm were then collected. The spectra obtained by collecting fluorescence emission between 500 and 599 nm upon excitation at 480 nm (Fig. 1) contain information about the extent of reactivation of the YPet component of the chimera (with CyPet either folded or not). The spectra between 450 and 600 nm following excitation at 430 nm can be decomposed into two parts: (i) a donor part that contains information about the yield of reactivation of the CyPet component of the chimera (with YPet either folded or not); and (ii) an acceptor part due to FRET that contains information about the yield of chimera molecules in which both the YPet and CyPet components have refolded. In addition, it was possible to obtain information about the extent of reactivation of the CyPet component of the chimera (with YPet either folded or not) from donor emission spectra between 450 and 500 nm upon excitation at 430 nm collected after cleavage of the chimera into its separate domains with proteinase K (Fig. 1). This cleavage reaction eliminates FRET from CyPet to YPet but does not affect the intrinsic fluorescence of YPet or CyPet (Fig. 1).

The above-mentioned emission intensities were converted (see Materials and Methods) into the relative populations of chimera molecules in which both the YPet and CyPet components are folded (CyY) and chimera molecules in which only the CyPet (Cy0) or YPet (0Y) domains are folded (see Fig. 2 for a scheme of the various possible species that can be observed). The population of chimera molecules in which neither domain is folded (00) was calculated from conservation of mass and assuming that at the highest ATP concentrations in our experiments no such molecules remain (see below). Plots of these populations as a function of ATP concentration are shown for the F44W wild-type variant (Fig. 3a) and the F44W/D155A mutant (Fig. 3b). It can be seen that the extent of regain of CyY, in the presence of the F44W wild-type variant, displays a monosigmoidal dependence on ATP concentration (Fig. 3a). By contrast, this extent of regain, in the presence of the F44W/D155A mutant, displays a bi-sigmoidal dependence on ATP concentration (Fig. 3b). Such a dependence was previously observed in the case of reactivation of other chimeras by this mutant[20] and it reflects the sequential nature of its ATP-induced allosteric transitions.[19] Biphasic behavior is, therefore, also observed in the decrease of the population of 00 with increasing ATP concentrations in the case of the mutant (Fig. 3b) but not the wild-type variant (Fig. 3a).

Concerted ATP-dependent release of both the CyPet and YPet domains in a chimera may result in folding of both of its domains. Hence, we designate this reaction by 00→CyY (Fig. 2). By contrast, when the ATP-induced allosteric transition of a ring is not concerted, as in the case of the mutant at relatively low ATP concentrations, then only one domain may be released and folded. These reactions that are designated by 00→Cy0 and 00→0Y occur in association with GroEL since the other domain remains attached (Fig. 2). It may be seen that, in the presence of the mutant, the amounts of Cy0 and 0Y initially increase with increasing ATP concentrations, thus reflecting non-concerted release, and then decrease (Fig. 3b). The decrease is expected since at high ATP concentrations all seven subunits switch from **t** to **r**,[19] thereby releasing most of the bound substrate. In the case of the wild-type variant, the initial increase in the amounts of Cy0 and 0Y with increasing concentrations of ATP is less pronounced (Fig. 3a), in agreement with a more concerted release mechanism. It should be noted that some Cy0 and 0Y remain even at the highest ATP concentrations employed. This was found to be due to incomplete substrate release and not to aggregation since the populations of Cy0 and 0Y decreased to almost zero (and the population of CyY increased in a corresponding manner) when, in addition to a high concentration (4 mM) of ATP, also GroES was added (Fig. 4). The elution profiles in Fig. 4 show that some 0Y and, in particular, Cy0 remains bound to GroEL and, thus, co-elutes with it when 4 mM ATP is added (Fig. 4c and d) and that complete release is reached when also GroES is added (Fig. 4e and f). The fact that more Cy0 than 0Y remains at the highest ATP concentrations employed (in particular in the case of the F44W/D155A mutant) reflects the relative folding yields of these domains by themselves in the presence of the mutant and wild-type GroEL variants (Fig. 5). Importantly, the total population of CyY, 0Y and Cy0 was found to be the same when 4 mM ATP were added either with or without GroES, thus validating our calculation of the population of 00 from conservation of mass that assumed that, at the highest concentrations of ATP in our experiments, it is negligible.

Surprisingly, substantial amounts of Cy0 and 0Y are found also prior to addition of ATP. Given that the amount of these species at high ATP concentrations is lower than in the absence of ATP indicates that some of the chimera molecules were initially bound to GroEL *via* one domain only with the other domain folded. The fact that there is more Cy0 than 0Y when no ATP is added (Fig. 3a and b) is consistent with the higher folding yields of the CyPet protein relative to the YPet protein in the absence of ATP (Fig. 5).

Two measures for assessing the cooperativity in release by GroEL were devised that circumvent the complications in data analysis that arise from the incomplete release at high ATP concentrations and from the fraction of molecules that are initially bound only *via* one

domain. The first measure is based on the expectation that, in the case of concerted release and folding (00→CyY), the decrease in the amount of 00 should match the increase in the amount of CyY. Hence, the decrease in the population of 00 at a given concentration i of ATP relative to its population in the absence of ATP ($[00]_0 - [00]_i$) is compared to the increase in the population of CyY at that concentration i of ATP relative to the population in the absence of ATP ($[CyY]_i - [CyY]_0$) by plotting the ratio of these changes as a function of ATP concentration (Fig. 6). In the case of the mutant, $([00]_0 - [00]_i)/([CyY]_i - [CyY]_0) > 1$, in the presence of ATP concentrations under ~1 mM, thus reflecting the non-concerted release and folding reactions 00→0Y and 00→Cy0 in which some of the 00 population is converted into species with only one folded domain (Cy0 or 0Y) (Fig. 6). In the case of the wild-type variant, $([00]_0 - [00]_i)/([CyY]_i - [CyY]_0) \approx 1$ at ATP concentrations under ~1 mM, in agreement with a concerted release mechanism. No difference between the wild-type variant and the mutant is observed at high ATP concentrations as expected since at these concentrations the allosteric switch is concerted in both of them. In fact, $([CyY]_i - [CyY]_0)$ is somewhat larger than $([00]_0 - [00]_i)$ at these concentrations because of the 0Y→CyY and Cy0→CyY reactions in which species with a single folded domain are released and converted into fully folded proteins. It should be noted, however, that $([CyY]_i - [CyY]_0)$ can also be equal to $([00]_0 - [00]_i)$ if the amounts of 0Y and Cy0 formed by 00→0Y and 00→Cy0 exactly match those consumed by the reactions 0Y→CyY and Cy0→CyY. Hence, a second measure for cooperativity was devised that takes into account also the concentrations of the 0Y and Cy0 species.

The second measure of cooperativity, Ω_i, is given by:

$$\Omega_i = \frac{[Cy0]_i [0Y]_i}{[CyY]_i [00]_i} \quad (1)$$

It is based on a thermodynamic cycle comprising the 00, Cy0, 0Y and CyY species (Fig. 2) that is analogous to a double-mutant cycle.[22] By analogy to coupling constants in other situations of thermodynamic linkage,[23] this measure compares for each concentration i of ATP the extent of release and folding of one domain when the other domain is folded and when its not (i.e. Cy0→CyY and 00→0Y, respectively, in the case of folding of the Y domain, and 0Y→CyY and 00→Cy0, respectively, in the case of folding of the Cy domain). If release and folding of the two domains is strongly coupled (i.e. concerted) then the main reaction will be 00→CyY, the concentrations of Cy0 and 0Y will be low and the value of Ω_i will decrease. If, on the other hand, the release of one domain inhibits the release of the other domain then the reactions will occur via the two other routes (00→Cy0→CyY and 00→0Y→CyY), the concentrations of Cy0 and 0Y will be high and the value of Ω_i will increase. Therefore, a lower value of Ω_i indicates that the reaction is more concerted. In Fig. 7, the ratio between the values of Ω_i for the wild-type variant and the mutant is plotted against ATP concentration (this ratio is a measure of the three-way coupling between folding of the two domains and the D155A mutation as in a triple-mutant cube[22]). It can be seen (Fig. 7) that the value of Ω_i is indeed lower for the wild-type variant than the mutant at low concentrations of ATP, thus reflecting the more concerted mechanism of the wild-type variant. The difference between the values of Ω_i for the wild-type variant and the mutant at the very low ATP concentrations is small owing to the presence of substantial amounts of Cy0 and 0Y prior to addition of ATP. The difference between the values of Ω_i for the wild-type variant and the mutant is small also at the high ATP concentrations when release and folding by both variants is concerted. These data are not shown owing to the large errors under those conditions.

Discussion

There is increasing appreciation that the problem of multi-domain protein folding deserves special consideration since inter-domain interactions may affect folding mechanisms and

kinetic partitioning between folding and mis-folding.[24] A neglected aspect of this problem is whether certain attributes of molecular chaperone mechanisms have evolved to specifically assist multi-domain protein folding. Previously, we speculated that ATP-induced sequential conformational changes in the eukaryotic chaperonin CCT may facilitate sequential protein substrate release and, as a result, domain-by-domain folding.[4] By contrast, we suggested that ATP-induced concerted conformational changes in the prokaryotic chaperonin GroEL facilitate all-or-none substrate release. The difference in these release mechanisms was proposed to reflect the need for a more efficient folding mechanism of multi-domain proteins that are more abundant in eukaryotic cells[25] and prone to misfold. A sequential release mechanism may have an adverse effect on single-domain protein folding since non-local interactions cannot form if not all parts of the protein are released from the chaperonin. It may, however, be beneficial for multi-domain proteins as it reduces the likelihood of formation of non-native interactions between domains. A recent two-dimensional lattice folding simulation study[26] has indeed shown that concerted conformational changes are more compatible with single-domain protein folding whereas double-domain proteins benefit more than single-domain proteins from sequential changes.

In the case of GroEL, it has been possible to test this hypothesis by comparing the folding of a multi-domain protein in the presence of wild-type GroEL and the F44W/D155A mutant[19] that undergo ATP-induced concerted and sequential conformational changes, respectively. In a previous study,[20] this test was carried out using a chimera in which a stringent GroES-dependent substrate, rhodanese, is fused to the non-stringent substrates mouse dihydrofolate reductase (mDHFR) or enhanced green fluorescent protein (EGFP). ATP-induced folding of the non-stringent part of the chimeras was found to occur in a stepwise fashion, with respect to ATP concentration, in the presence of the F44W/D155A mutant but not the wild-type variant.[20] In other words, the dependence on ATP concentration of the folding yield of the non-stringent part of the chimeras was found to biphasic in the case of the mutant that undergoes a sequential allosteric switch (e.g. $t_7 \rightarrow t_3r_4 \rightarrow r_7$) and sigmoidal in the case of the wild-type variant that undergoes a concerted switch ($t_7 \rightarrow r_7$).

In this study, a different test of this hypothesis was devised that involves using a chimera, CyPet-YPet, where both domains can fold upon addition of ATP. Hence, we were able to examine whether (i) release of one domain tends to be accompanied by release of the other domain or (ii) release of the two domains is not coupled. Based on the fluorescence properties of the individual domains and FRET between the two domains, it has been possible to show here that the degree of concertedness of the ATP-induced conformational change in GroEL correlates with the extent of concerted release and folding of a protein substrate. In other words, concerted release and folding (00→CyY) is more favored in the presence of the wild-type variant that undergoes a concerted allosteric switch than in the case of the mutant that displays ATP-induced broken symmetry and undergoes a sequential allosteric transition. It should be noted that concerted release from the wild-type variant might still result in a sequential folding pathway that will require transient kinetic methods to be resolved. Our results clearly show, however, that a break-in-symmetry favors sequential release and, as a result, sequential folding. A sequential allosteric switch, as found in CCT, provides a mechanism, therefore, for forcing a sequential folding pathway even in cases that it is not encoded in the sequence of the protein substrate. The mechanism of conformational switching is an aspect of allosteric regulation which is of particular importance for molecular machines since their efficiencies are path dependent, i.e. their output depends not only on inter-conversion between different allosteric (functional) states but also on the pathways by which they inter-convert. Our results, thus, establish a connection between a key allosteric property of this molecular machine and its biological function in assisting folding.

Materials and Methods

Molecular biology

The gene coding for the CyPet-YPet fusion protein[21] in pLNCX (Clontech) was amplified using the oligonucleotides: FOR, 5′-GCAAGCCATATGCGACGGTACCGCGGGC-3′; and BACK, 5′-CGAACGAAGCTTATCGATGTTGGCCGC-3′. These oligonucleotides introduce *Nde*I and *Hind*III restriction sites (underlined) that flank the 5′ and 3′ ends of the gene, respectively. The PCR product was digested with *Nde*I and *Hind*III and then inserted into the vector pET21a(+)/His (Novagen) previously digested with the same enzymes. A stop codon between the C-terminus of YPet in the chimera and the His_6-tag was removed using the QuickChange kit (Stratagene) and the oligonucleotides: FOR, 5′-GCATGAACGAGCTCTATAAG*AAAGCGGCCAACATCGATAAGC-3′; BACK, 5′-GCTTATCGATGTTGGCCGCTTT*CTTATAGAGCTCGTTCATGC-3′ (the asterisks indicate the position of the deleted nucleotide). This resulted in addition of the sequence SGQHRKLAAALE between the chimera's C-terminus and the His_6-tag. The sequence of the entire gene was verified by DNA sequencing.

Protein expression and purification

The chimera protein was obtained as follows. *E. coli* BL21(DE3) pLys cells harboring the pET21a(+)/chimera-His plasmid were grown overnight at 37 °C in Luria broth (LB) containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The overnight culture was diluted 1:100 in LB containing the same antibiotics and the cells were then grown at 37 °C until an O.D. of 1.0 was reached. The temperature was then lowered to 20 °C and 1 mM IPTG was added to initiate expression. The cells were grown for an additional 10 hours and then centrifuged at 3,000 g for 15 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 10 mM imidazole and 1 mM β–mercaptoethanol (buffer A). It was then centrifuged as before, stored at −80 °C until use and then resuspended in buffer A containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The cells were then disrupted by sonication and the lysate was clarified by centrifugation at 30,000 g for 30 min. The supernatant (70 ml) was loaded on a 5 ml HisTrap HP column (Pharmacia) that was charged with 0.1 M $NiSO_4$ as described by the manufacturer, and eluted with a gradient of 10-500 mM imidazole (60 ml at 1 ml/min) in buffer A. Yellow/green-colored fractions (that also displayed fluorescence at 530 nm upon excitation at 430 nm) that eluted at 200 mM imidazole were analysed by SDS-PAGE. The appropriate fractions were combined, transferred into 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) (buffer B) using a PD-10 desalting column (Pharmacia) and then concentrated using Vivaspin 20 (Sartorius). They were then loaded on a MonoQ column (Pharmacia) pre-equilibrated with buffer B and eluted with a gradient of 0-600 mM NaCl (60 ml at 1 ml/min) in buffer B. Yellow/green-colored fractions that eluted at 140 mM NaCl were analysed by SDS-PAGE and the appropriate fractions were then combined, concentrated and loaded on a Superdex 75 gel-filtration column (Pharmacia) that had been pre-equilibrated with buffer B. Chimera-containing fractions were analysed as above and the appropriate fractions were combined, concentrated and divided into aliquots that were snap-frozen and stored at −80 °C.

The CyPet and YPet proteins were expressed in *E. coli* DH5α cells harboring the pBAD33 plasmid that contains the gene for either CyPet-His_6 or YPet-His_6 as described.[21] Overnight cultures of these cells grown at 37 °C in LB containing 34 μg/ml chloramphenicol were diluted 1:100 in the same medium and the cells were then grown at 37 °C until an O.D. of 0.5 was reached. The temperature was then lowered to 20 °C and 0.2% (w/v) L-arabinose was added to initiate expression. The cells were grown for an additional 10 hours (0.2% L-arabinose was added again after 3.5 hours) and then centrifuged at 3,000 g for 15 min. Clarified lysate was obtained and purified on a 1 ml HisTrap column as described above for the chimera. Yellow/

green-colored fractions that eluted at 175 mM imidazole were analysed by SDS-PAGE. The appropriate fractions were combined, transferred into buffer B, concentrated and then stored at −80 °C as described above for the chimera. Protein concentrations were determined using the Bradford assay and BSA as a standard. The F44W and F44W/D155A GroEL mutant proteins were obtained as described before.[27]

Refolding assays

The chimera (50 μM) was denatured in 60 mM HCl (final pH of 1.5) and then diluted 1:100 in a siliconized test tube containing 50 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT (refolding buffer) and 1 μM of GroEL (F44W or F44W/D155A mutants) at 25 °C. The refolding mixture was incubated for 1 hour until no more spontaneous folding could be observed and GroEL-assisted folding was then initiated by adding ATP at the indicated concentration. Reactivation of chimera molecules in which both fluorescent proteins are folded and of the individual domains CyPet and YPet in the chimera (with the other component either folded or not) was monitored by recording the appropriate emission spectra two hours after the addition of ATP. The spectra were analyzed as described below. The total amount of folded CyPet was also determined after overnight cleavage with 20 μM proteinase K at room temperature.

Data analysis

Fluorescence emission spectra of the reaction mixtures at different ATP concentrations were recorded using an ISS PC1 spectrofluorometer and then corrected for the wavelength dependent response of the instrument's detection system as specified by the manufacturer. The emission spectrum of the acceptor, YPet, upon direct excitation at 480 nm, was integrated between 500 and 599 nm and is designated by I^A_{480}. The emission spectra, upon excitation at 430 nm, were decomposed into the respective donor (CyPet) and acceptor parts, I^D_{430} and I^A_{430}, using the spectra of the individual proteins (Fig. 1, inset). The intensities I^A_{480}, I^D_{430} and I^A_{430} at each ATP concentration were determined in triplicate, normalized to the average of the maximal intensities (obtained at the highest ATP concentrations employed) and then averaged. These average emission intensities were then converted to be on a scale that is free of variations in quantum efficiencies and excitation strengths, as follows: $I_1 = I^D_{430}/Q_D$; $I_2 = I^A_{480}\varepsilon^D_{430}R_{D/A}/Q_A\varepsilon^A_{430}$ and $I_3 = I^A_{430}/Q_A - (I^A_{480}/Q_A)D$. Here, Q_D and Q_A designate the donor and acceptor quantum efficiencies, ε^D_{430} and ε^A_{480} are the extinction coefficients of the donor at 430 nm and the acceptor at 480 nm, D is the fraction of acceptor emission due to direct excitation at 430 nm (as opposed to FRET) and $R_{D/A}$ is the ratio of lamp intensities at 430 and 480 nm. I_3 is corrected for direct acceptor excitation at 430 nm. Given I_1, I_2 and I_3 one can derive expressions (to within a constant factor) for the populations of chimera molecules with only the YPet domain folded (0Y), only the CyPet domain folded (Cy0) and with both domains folded (CyY), as follows: [Cy0] = I_1 - I_3(1-E)/E; [0Y] = I_2 − I_3/E; and [CyY] = I_3/E where E is the FRET efficiency. The emission intensity of the donor after proteinase K cleavage, $I_4(=I^D_{430}(\text{cut})/Q_D)$, corresponds to $[Cy]_{total}$ = [Cy0] + [CyY]. Hence, a consistency check for our calculations and experimental accuracy is obtained by determining whether $I_4 = I_1 + I_3$.

The values of ε^D_{430} and ε^A_{480} were determined from the slope of plots of the absorbance as a function of concentration (Beer's law) and found to be 18.3 (± 0.9) and 15.6 (± 1.2) mM^{-1} cm^{-1}, respectively. The values of Q_D and Q_A were determined from the slopes of the integrated emission intensity vs. absorbance of the donor (relative to fluorescein in 0.1 M NaOH) and acceptor (relative to rhodamine 6G in ethanol) and found to be 0.66 (± 0.07) and 0.22 (± 0.03), respectively, after correcting for the refractive indices of the media.[28] The value of E, found to be 0.65, was determined using the relation $E = 1 - \langle\tau_{CyY}\rangle/\langle\tau_{Cy}\rangle$, where $\langle\tau_{Cy}\rangle$ and $\langle\tau_{CyY}\rangle$ are

the respective average fluorescence life-times of CyPet alone and in the chimera measured using a Jobin-Yvon FluoroCube life time spectrofluorometer. The value of D, found to be 0.08, was determined from the ratio of corrected and integrated emission intensities upon excitation at 430 and 480 nm. The value of the correction factor, $R_{D/A}$, was determined using a concentrated solution of rhodamine B as a quantum counter and found to be 1.25.

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References

1. Horwich AL, Weber-Ban EU, Finley D. Chaperone rings in protein folding and degradation. Proc Natl Acad Sci USA 1999;96:11033–11040. [PubMed: 10500119]
2. Patel SS, Donmez I. Mechanisms of helicases. J Biol Chem 2006;281:18265–18268. [PubMed: 16670085]
3. Horovitz A, Willison KR. Allosteric regulation of chaperonins. Curr Opin Struct Biol 2005;15:646–651. [PubMed: 16249079]
4. Rivenzon-Segal D, Wolf SG, Shimon L, Willison KR, Horovitz A. Sequential ATP-induced allosteric transitions of the cytoplasmic chaperonin containing TCP-1 revealed by EM analysis. Nat Struct Mol Biol 2005;12:233–237. [PubMed: 15696173]
5. Martin A, Baker TA, Sauer RT. Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. Nature 2005;437:1115–1120. [PubMed: 16237435]
6. Thirumalai D, Lorimer GH. Chaperonin-mediated protein folding. Annu Rev Biophys Biomol Struct 2001;30:245–269. [PubMed: 11340060]
7. Saibil HR, Horwich AL, Fenton WA. Allostery and protein substrate conformational change during GroEL/GroES-mediated protein folding. Advan Protein Chem 2002;59:45–72. [PubMed: 11868280]
8. Horwich AL, Fenton WA. The role of ATP in directing chaperonin-mediated polypeptide folding. The Enzymes 2003;23:399–433.
9. Lin Z, Rye HS. GroEL-mediated protein folding: making the impossible, possible. Crit Rev Biochem Mol Biol 2006;41:211–239. [PubMed: 16849107]
10. Braig K, Otwinowski Z, Hegde R, Boisvert DC, Joachimiak A, Horwich AL, Sigler PB. The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. Nature 1994;371:578–586. [PubMed: 7935790]
11. Schmidt M, Buchner J, Todd MJ, Lorimer GH, Viitanen PV. On the role of groES in the chaperonin-assisted folding reaction. Three case studies. J Biol Chem 1994;269:10304–10311. [PubMed: 7908292]
12. Yifrach O, Horovitz A. Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL. Biochemistry 1995;34:5303–5308. [PubMed: 7727391]
13. Yifrach O, Horovitz A. Allosteric control by ATP of non-folded protein binding to GroEL. J Mol Biol 1996;255:356–361. [PubMed: 8568880]
14. Yifrach O, Horovitz A. Mapping the transition state of the allosteric pathway of GroEL by protein engineering. J Amer Chem Soc 1998;120:13262–13263.
15. Shiseki K, Murai N, Motojima F, Hisabori T, Yoshida M, Taguchi H. Synchronized domain opening motion of GroEL is essential for communication between the two rings. J Biol Chem 2001;276:11335–11338. [PubMed: 11139586]
16. Ma J, Sigler PB, Xu Z, Karplus M. A dynamic model for the allosteric mechanism of GroEL. J Mol Biol 2000;302:303–313. [PubMed: 10970735]
17. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: a plausible model. J Mol Biol 1965;12:88–118. [PubMed: 14343300]

18. Yifrach O, Horovitz A. Transient kinetic analysis of adenosine 5′-triphosphate binding-induced conformational changes in the allosteric chaperonin GroEL. Biochemistry 1998;37:7083–7088. [PubMed: 9585518]

19. Danziger O, Rivenzon-Segal D, Wolf SG, Horovitz A. Conversion of the allosteric transition of GroEL from concerted to sequential by the single mutation Asp-155→Ala. Proc Natl Acad Sci USA 2003;100:13797–13802. [PubMed: 14615587]

20. Kipnis Y, Papo N, Haran G, Horovitz A. Concerted ATP-induced allosteric transitions in GroEL facilitate release of protein substrate domains in an all-or-none manner. Proc Natl Acad Sci USA 2007;104:3119–3126. [PubMed: 17360617]

21. Nguyen AW, Daugherty PS. Evolutionary optimization of fluorescent proteins for intracellular FRET. Nat Biotechnol 2005;23:355–360. [PubMed: 15696158]

22. Horovitz A, Fersht AR. Strategy for analysing the co-operativity of intramolecular interactions in peptides and proteins. J Mol Biol 1990;214:613–617. [PubMed: 2388258]

23. Wyman, J.; Gill, SJ. Binding and Linkage. University Science Books; Mill Valley, CA: 1990.

24. Han JH, Batey S, Nickson AA, Teichmann S, Clarke J. The folding and evolution of multidomain proteins. Nat Rev Mol Cell Biol 2007;8:319–330. [PubMed: 17356578]

25. Netzer WJ, Hartl FU. Recombination of protein domains facilitated by co-translational folding in eukaryotes. Nature 1997;388:343–349. [PubMed: 9237751]

26. Jacob E, Horovitz A, Unger R. Different mechanistic requirements for prokaryotic and eukaryotic chaperonins: a lattice study. Bioinformatics 2007;23:i240–i248. [PubMed: 17646302]

27. Danziger O, Shimon L, Horovitz A. Glu257 in GroEL is a sensor involved in coupling polypeptide substrate binding to stimulation of ATP hydrolysis. Protein Sci 2006;15:1270–1276. [PubMed: 16672234]

28. Lakowicz, JR. Principles of Fluorescence Spectroscopy. 2. Kluwer Academic/Plenum Publishers; New-York: 1999.

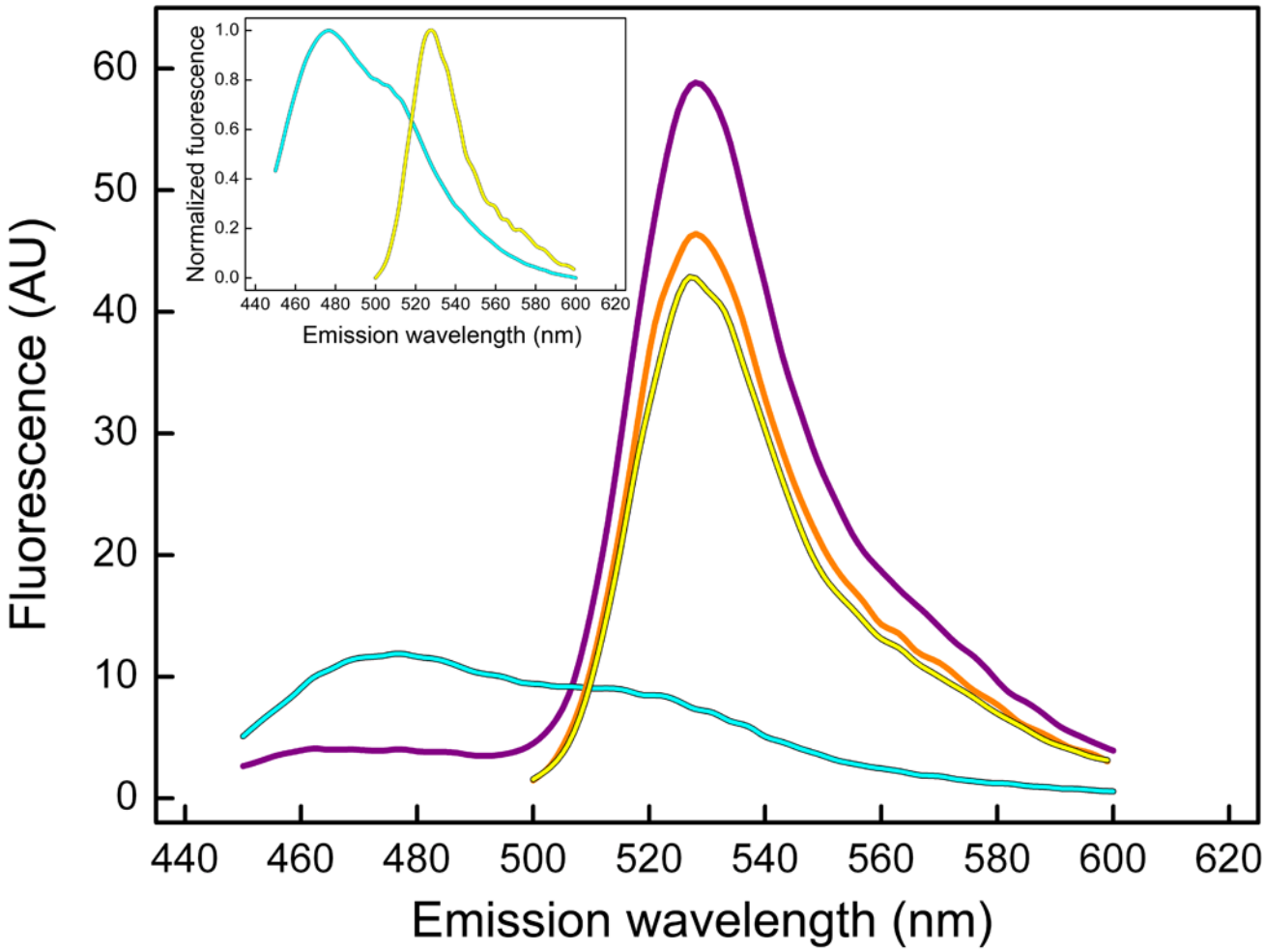

Fig. 1.

Emission spectra of the cyan and yellow fluorescent proteins, CyPet and YPet, and of the CyPet-YPet chimera. Excitation of the chimera at 430 nm leads to emission by YPet owing to FRET (magenta). The FRET is abolished by proteinase K cleavage of the chimera into its separate domains and CyPet fluorescence is, therefore, observed upon excitation at 430 nm (cyan). The cleavage does not affect the intrinsic fluorescence of the domains (thus indicating their resistance to proteolysis) as may be seen by comparing the fluorescence, upon excitation at 480 nm, of YPet in the chimera (orange) with that of YPet derived from cleaving the chimera (yellow). The inset shows the emission spectra of CyPet (cyan) and YPet (yellow) that were expressed separately, upon excitation at 430 and 480 nm, respectively. All spectra were corrected for wavelength dependence of excitation intensities and the instrument spectral response.

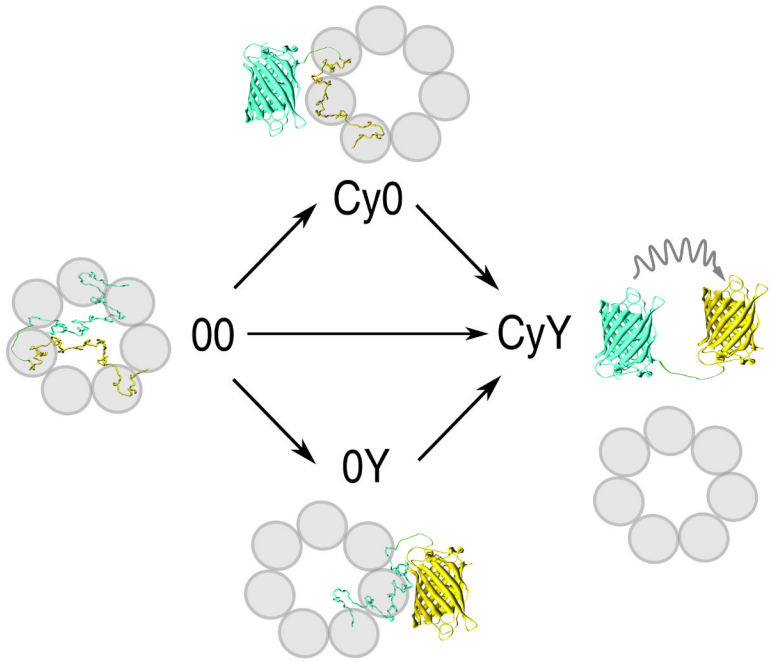

Fig. 2.

A cycle showing the different folding transitions of the chimera. The GroEL- bound chimera in which both the CyPet and YPet domains are unfolded (00) may be released by ATP in a concerted fashion that leads to the folding of both domains (00→CyY). Alternatively, only the CyPet or YPet domains may be released and folded as designated by the reactions 00→Cy0 and 00→0Y, respectively. In these cases, the chimera remains bound to GroEL (depicted schematically as a heptameric ring) *via* its unfolded domain. Models of the four states of the chimera are shown that indicate their respective spectroscopic properties relevant to this study. The Cy0 and 0Y species have the intrinsic fluorescence of the CyPet (cyan) and YPet (yellow) domains, respectively, whereas CyY has both and is the only species with FRET between the two domains, as indicated by the wavy arrow. The 00 species lacks all of these properties.

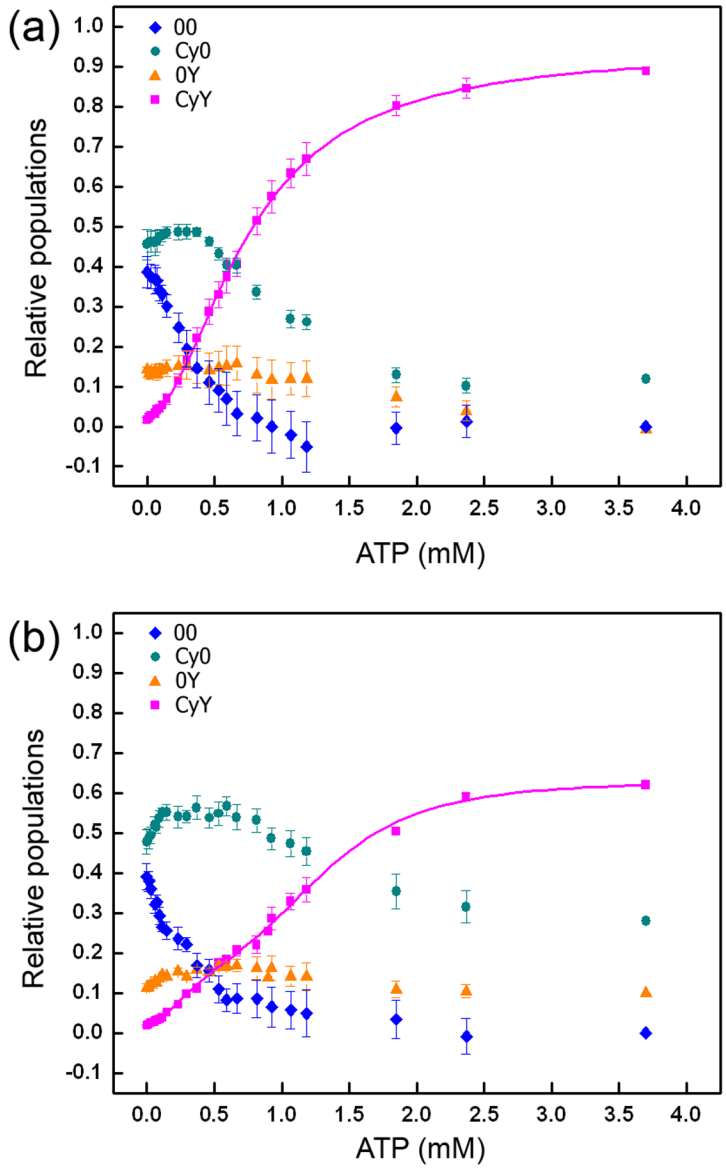

Fig. 3.

Fractions of the total chimera population of chimeras in which both, only one or neither of the CyPet and YPet domains are folded as a function of ATP concentration. The relative populations of chimeras molecules in which both the YPet and CyPet components are folded (CyY; magenta), only the CyPet (Cy0; cyan) or YPet (0Y; orange) domains are folded and neither domain is folded (00; blue), in the presence of the wild type variant of GroEL (a) and the F44W/D155A mutant (b) at different ATP concentrations, were calculated as described in Materials and Methods. The data of chimera molecules in which both the YPet and CyPet components are folded (CyY) were fitted to the Hill equation, in the case of the wild-type variant, and to a double-Hill equation in the case of the mutant. Each experiment was carried out in triplicate and standard errors of the mean are shown.

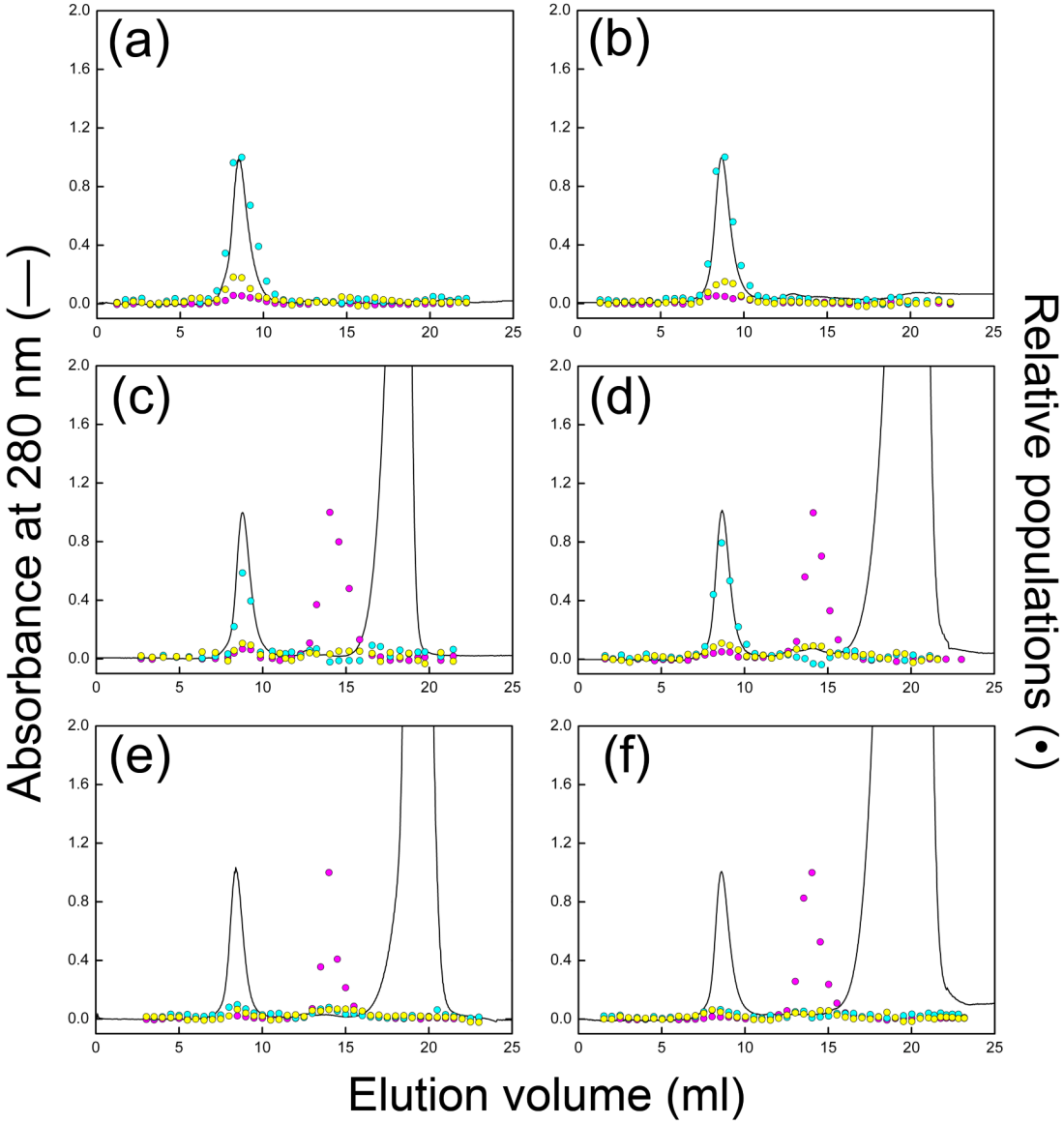

Fig. 4.

Gel filtration of the products of GroEL-assisted reactivation of the denatured CyPet-YPet chimera. (a, b) Elution profiles of the denatured chimera in complex with wild-type GroEL (a) or the F44W/D155A mutant (b) in the absence of ATP and GroES. (c, d) Elution profiles of the reaction products of reactivation of the denatured chimera in the presence of wild-type GroEL (c) or the F44W/D155A mutant (d) and 4 mM ATP but without GroES. (e, f) Elution profiles of the reaction products of reactivation of the denatured chimera in the presence of wild-type GroEL (e) or the F44W/D155A mutant (f), 4 mM ATP and 2 μM GroES. The elution profiles were obtained by measuring the absorbance at 280 nm (black line) and the appropriate emission spectra needed to determine the populations of CyY (magenta), Cy0 (cyan) and 0Y (yellow). The populations in each panel were normalized separately relative to each other. The material detected by the absorbance at 280 nm that elutes after about 8 and 19 ml corresponds to GroEL and ATP, respectively. These reactivation reactions were carried out at 25 °C in 1 ml of refolding buffer containing 1 μM GroEL and 0.5 μM denatured chimera. The fluorescence

emission of each reaction was monitored and when it reached a maximum the sample was concentrated to less than 100 μL using a Microcone ultrafiltration device with a 10 kDa cut-off filter (in all cases the flow-through had no fluorescence) and loaded onto a Superdex 200 HR 10/30 gel-filtration column. The gel-filtration was carried out in refolding buffer at room temperature at a flow rate of 0.5 ml/min.

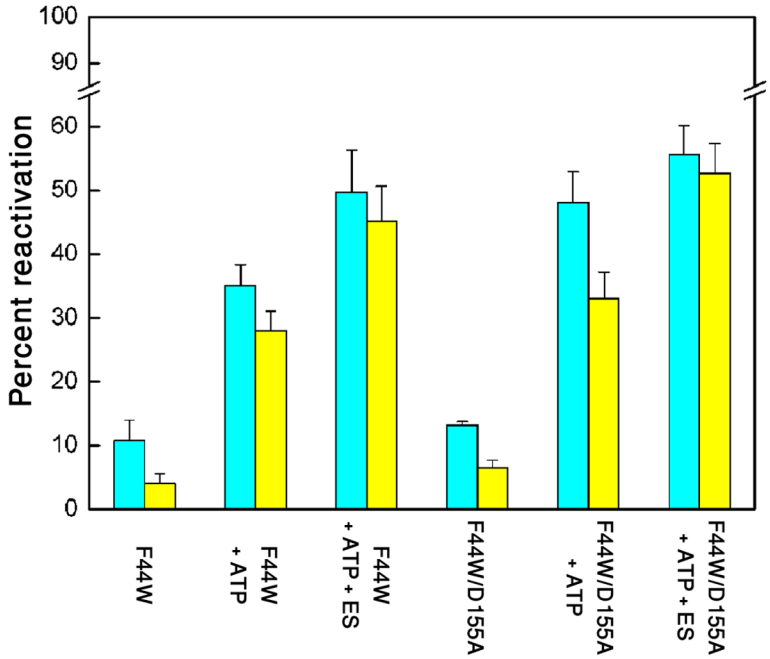

Fig. 5.

Reactivation of denatured CyPet and YPet proteins that are not fused to each other. Reactivation of the individual CyPet (cyan) and YPet (yellow) proteins following their denaturation was carried out at 25 °C in refolding buffer containing 1 μM GroEL (F44W or F44W/D155A) with or without 2 μM GroES and/or 4 mM ATP. The yields of reactivation are expressed relative to the total amount of denatured material. Each experiment was repeated three times and standard deviations were determined. See Materials and Methods for more details.

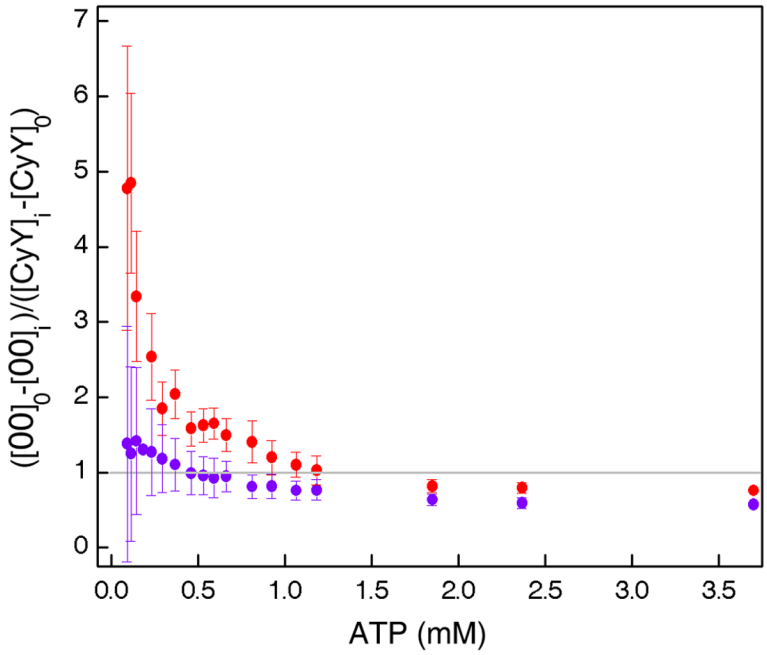

Fig. 6.

Plots of the ratio between the change in population of chimera molecules in which the CyPet and YPet domains are both unfolded (00) and the change in population of chimera molecules in which both domains are folded (CyY), at different concentrations of ATP. The ratio between the decrease in the population of 00 and the increase in the population of CyY, in the presence of the wild-type (blue) or mutant (red) variants of GroEL, is plotted as a function of ATP concentration. The changes in the populations of 00 and CyY at each concentration i of ATP are calculated relative to the populations in the absence of ATP ($([00]_0 - [00]_i)$ and $([CyY]_i - [CyY]_0)$, respectively). The gray line with a slope of zero indicates when the values of $[CyY]_i - [CyY]_0$ and $[00]_0 - [00]_i$ exactly match.

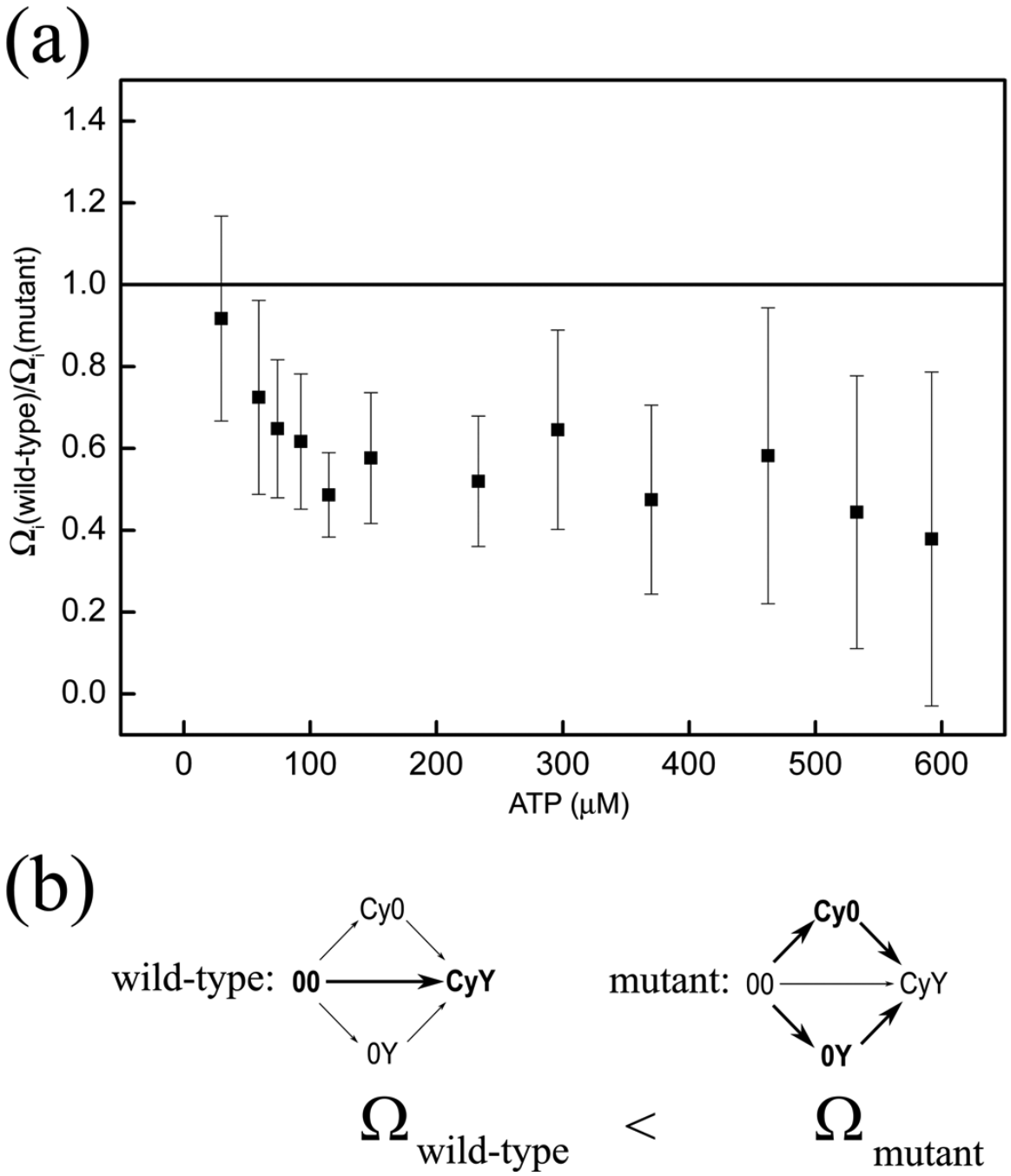

Fig. 7.

Plot of the ratio of the coupling constant-type measures for cooperativity in the release and folding of the chimera components. The measures Ω_i(wild-type) and Ω_i(mutant) quantify the extent of concertedness in the release and folding of the CyPet and YPet components of the chimera by the respective wild-type and mutant GroEL variants at different concentrations i of ATP as described in the text (Eq. (1)). The straight line with a value of 1 is intended to highlight the fact that the values of the ratio Ω_i(wild-type)/Ω_i(mutant) with error are below 1 (a). In the case of sequential release, the populations of Cy0 and 0Y shown in bold are dominant (b, right cycle) whereas in the case of concerted release the populations of 00 and CyY shown in bold are larger (b, left cycle). The value of Ω_i is, therefore, smaller in the latter case when

release is more concerted. Hence, Ω_i(wild-type)/Ω_i(mutant) < 1 indicates that the release mechanism of the wild-type variant is more concerted than that of the mutant.