Elucidation of Steps in the Capture of a Protein Substrate for Efficient Encapsulation by GroE *

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We have identified five structural rearrangements in GroEL induced by the ordered binding of ATP and GroES. The first discernable rearrangement (designated \( T \rightarrow R_1 \)) is a rapid, cooperative transition that appears not to be functionally communicated to the apical domain. In the second \( (R_1 \rightarrow R_2) \) step, a state is formed that binds GroES weakly in a rapid, diffusion-limited process. However, a second optical signal, carried by a protein substrate bound to GroEL, responds neither to formation of the \( R_2 \) state nor to the binding of GroES. This result strongly implies that the substrate protein remains bound to the inner walls of the initially formed GroEL-GroES cavity, and is not yet displaced from its sites of interaction with GroEL. In the next rearrangement \( (R_2 \rightarrow \text{GroES} \rightarrow R_3 \rightarrow \text{GroES}) \), the strength of interaction between GroEL and GroES is greatly enhanced, and there is a large and coincident loss of fluorescence signal intensity in the labeled protein substrate, indicating that there is either a displacement from its binding sites on GroEL or at least a significant change of environment. These results are consistent with a mechanism in which the shift in orientation of GroEL apical domains between that seen in the apo-protein and stable GroEL-GroES complexes is highly ordered, and transient conformational intermediates permit the association of GroES before the displacement of bound polypeptide. This ensures efficient encapsulation of the polypeptide within the GroEL central cavity underneath GroES.

The function of the GroE molecular chaperone is to enhance the efficiency of protein folding, whether the substrate is a newly synthesized protein chain or a denatured molecule that has unfolded in response to environmental conditions (1–4). This dual role means that GroE is expressed constitutively to fulfill the former function and is inducible during heat-shock, when its concentration in the Escherichia coli cytoplasm is raised 5-fold (5). The ability of this chaperone to increase the productive folding yield, irrespective of either the sequence of the chain or the final shape of the folded substrate, is mechanismically intriguing, as is the coupling of this folding activity with the hydrolysis of ATP.

The GroE chaperone comprises two protein assemblies. The largest, GroEL (sometimes referred to as Cpn60, a chaperonin with a subunit molecular mass of 60 kDa), is constructed from fourteen identical subunits arranged in two rings, stacked back-to-back, to form two large cavities separated by a central septum but with wide openings allowing access to the environment (6). These basket-like cavities are capable of encompassing an unfolded protein substrate of \( \sim 60 \) kDa chain molecular mass (7, 8).

The second protein component, GroES (sometimes referred to as Cpn10, a chaperonin with a subunit molecular mass of 10 kDa), is a single ring of seven members that forms a dome-like structure of a diameter that is matched to the width of the GroEL opening (9). In the presence of adenine nucleotides, GroES binds preferentially to one of the GroEL rings to act as a lid that can close off one of the central cavities to create a capsule (10, 11). It is within this capsule, or cage, that substrate proteins are able to fold, relatively uncumbered by interactions with the protein surfaces that constitute its walls (7, 8). More salient to the biological function of the machine, the encapsulated protein chain is not free to form interactions with other protein molecules in a similarly unfolded state, a process that might otherwise lead to the formation of inactive aggregates (12–16). If these are not recoverable, other than by hydrolytic breakdown and de novo re-synthesis, it is a waste of the synthetic efforts of the cell. Hence, preventing irreversible aggregation, albeit at the cost of ATP hydrolysis, is a useful process.

GroEL, GroES, and ATP are the fundamental requirements for the complete, energy-transducing ATPase in the assisted-folding reaction (12). Our current understanding of the principle steps that constitute the reaction cycle are summarized in Fig. 1. As far as is currently understood, there are five rules that govern the operation of the cycle: (a) the GroES co-protein cannot bind to a ring devoid of nucleotide (17); (b) GroES binds more rapidly to an ATP-occupied ring than to one occupied by ADP (18–20); (c) the binding of seven ATP molecules to one ring leads to a weakening of binding to the other, i.e. there is negative homotropic cooperativity between the rings (18, 21); (d) mixed complexes with one ring occupied by ATP and the other by ADP are stable, but cannot undergo the hydrolytic step owing to ADP on one ring acting as a non-competitive inhibitor of hydrolysis on the other (22); and (e) the binding of GroES to an ATP-occupied ring commits the nucleotide to hydrolysis (23).

Much previous work shows that the substrate proteins are transiently ensnared in the GroES-capped cavity during this
ATPase cycle and have a dwell time of tens of seconds in which the protein substrate has a probability of folding dictated by the rate constant of the spontaneous process (24, 25). Because encapsulation cannot rely upon the random chance of an unfolded substrate happening to be in the cavity at the same time that the GroES lid comes on, there must be a mechanism by which the substrate is concentrated in the hole before the lid can provide the means of forming the cage. The structural picture that we have at the moment, derived from both x-ray and electron microscopy techniques, shows that in static or, at least, long-lived species, the binding of GroES and substrate are rather exclusive, i.e. they share binding surfaces (10, 26–28). Taken at face value this means that the protein substrate has to dissociate before GroES can bind. If this is true, then there must be a conformational change that obligatorily displaces the protein substrate, which then has no time to diffuse from the locale before GroES rapidly caps the cavity and thus achieves encapsulation. However, an alternative mechanism can be proposed that proceeds through an intermediate state, as yet unseen by structural methods nor inferred from kinetics, in which the protein substrate remains bound to the walls of GroEL at the same time that GroES associates with its seven apical domains. This pathway postulates that there is a transient conformational intermediate state for GroEL in which the binding of substrate protein chain and GroES are not mutually exclusive, i.e. GroES binds before displacement of the substrate. There then must be a further step in which the substrate is shed from its binding site on the apical domain and allowed to fold unhindered.

It is difficult to distinguish between these two plausible mechanisms except by using time-resolved techniques that can report specific events in the millisecond time range. Previous work on the structural rearrangement of GroEL induced by the binding of ATP shows that the picture is complex, even in the absence of GroES, with four distinct transitions before the nucleotide is hydrolyzed (29–31). These re-organizations of GroEL are spread over the several milliseconds to hundreds of milliseconds time-scales and are thus in the time window of stopped-flow techniques. In an attempt to resolve the molecular events that constitute the encapsulation phase of the GroE chaperone mechanism we have used fast-mixing methods combined with fluorescence labeling to determine the effect of GroES on the ATP-induced structural rearrangements in GroEL, including the point at which GroES associates. This is combined with time-resolved data that monitor the response of a label attached to the protein substrate when ATP-induced structural rearrangements occur in GroEL. The combination of these two lines of enquiry is used to correlate the dynamics of the protein substrate and of GroES.

EXPERIMENTAL PROCEDURES

Standard Conditions—The standard reaction buffer in all experiments, except where stated otherwise, was 50 mM triethanolamine hydrochloride, pH 7.5, 50 mM KCl, and 20 mM MgCl₂.

Proteins and Reagents—The W485-GroEL mutant was a kind gift from Dr. Peter Lund (Birmingham, UK). The mutant GroEL and GroES were purified as described previously (30, 18). Reduced carboxymethylated α-lactalbumin was obtained from Sigma. ATP and ADP were obtained from Roche Applied Science. Nucleotide concentrations were confirmed spectrophotometrically using a molar extinction coefficient of 15,300 M⁻¹ cm⁻¹ for adenine at 260 nm. All other reagents were obtained from BDH and were analytical grade.

Fluorescent Labeling of CM-LA—A 10 mM stock solution of 5-((dimethylamino)-1-naphthalenesulfonyl chloride (dansyl chloride) in acetonitrile was added to 100 μM CM-LA³ in phosphate buffer, pH 7.5, at a ratio of 1:10. This pH was chosen to selectively label the α-amino group at the N terminus of CM-LA rather than the ε-amino group of lysine. The reaction was allowed to proceed for 10 min in the dark before being dialyzed overnight against 100 volumes of 50 mM Tris, pH 7.5, and 10 mg/ml activated charcoal. The extent of labeling was determined by absorbance using a molar extinction coefficient of 3400 M⁻¹ cm⁻¹ at 335 nm. On average one dansyl group was attached per CM-LA polypeptide.

Chemical Modification of the Tryptophan Residues in CM-LA—2-Hydroxy-5-nitrobenzyl bromide (HNBB, also known as Koshland Reagent I) reacts in neutral and acid solutions with only tryptophan residues (37) modifying the indole group of the protein substrate when ATP-inhibited structural rearrangements occur in GroEL. The combination

³ The abbreviations used are: CM-LA, reduced and carboxymethylated α-lactalbumin; HNBB, 2-hydroxy-5-nitrobenzyl bromide.
stock of HNBB was made in 5% dried acetone before mixing with an equal volume of 200 μM CM-LA in 10 mM sodium phosphate at pH 6.0 in the dark for 2 h. The mixed sample was then dialyzed against 200 volumes of 10 mM sodium phosphate, pH 6.0, in the dark. HNBB activity was confirmed by the loss of tryptophan fluorescence upon excitation at 295 nm.

Stopped-flow Time-resolved Tryptophan Fluorescence Measurements—All stopped-flow experiments were performed in the standard buffer using an Applied Photophysics SX-17MV stopped-flow fluorometer. W485-GroEL was excited using monochromatic light at 295 nm, and the resulting fluorescence was selected with a WG320 filter which cuts off all light below 320 nm. All reactions were performed at 25 °C, and at least three transients were averaged for any data point. Fluorescence measurements of the dansyl-CM-LA were made using an excitation wavelength of 340 nm and fluorescence emission selected using a WG420 filter, which cuts off light below 420 nm.

Analytical Methods—All data fitting was carried out using the least-squares method of Marquardt (43) using the Grafit 5.0 fitting program (Erithacus software). The quadratic tight ligand binding equation (Equation 1) used in Fig. 5 is,

\[
F = \frac{(E_0 + K_d + L)}{2E_0} \bigg( \sqrt{(E_0 + K_d + L)^2 - 4E_0L} \bigg) + F_{\text{min}} - F_{\text{max}} \quad \text{(Eq. 1)}
\]

where \( F \) is the measured fluorescence, \( E_0 \) is the initial concentration of ligand binding sites, \( K_d \) is the dissociation constant, \( L \) is the total ligand concentration, and \( F_{\text{max}} \) and \( F_{\text{min}} \) are the maximum and minimum fluorescence intensities observed, respectively. All parameters during the fitting procedures were allowed to float where possible, although it should also be noted that where the magnitude of the rate constants for successive phases are similar, then the determination of both rate constants and fluorescence amplitudes are not accurate. This was particularly the case when determining the rate constants and amplitudes for the \( R_3 \to R_3^* \) and \( R_3^* \to R_4 \) transitions at low concentrations of ATP. This affects the degree of accuracy of the value of \( K_{12} \) reported in Fig. 5B.

RESULTS

Discrete Events in the Ordered Addition of ATP and GroES to GroEL—The intrinsic fluorescence of an engineered tryptophan (Trp-485) in GroEL can be used to report conformational events induced by the binding of ATP (30, 31). This experiment was performed by rapidly mixing W485-GroEL (hereafter referred to just as GroEL for simplicity) with a solution of ATP using a stopped-flow apparatus and recording time-resolved changes in fluorescence (Fig. 2A, GroEL plus ATP). To investigate the effect of GroES on this reaction the experiment was repeated, this time challenging GroEL with a mixture of ATP and GroES (Fig. 2A, GroEL plus GroES plus ATP). In both reactions the transients observed are complex. ATP alone induces three, easily resolved kinetic phases (two fluorescence quenches and a subsequent fluorescence enhancement), whereas ATP and GroES together induce four kinetic phases (three quenches and a slower enhancement). In both cases they are also preceded...
by a very rapid signal enhancement, which represents the cooperative $T \rightarrow R$ transition but which is not fully resolvable at the ATP concentration and temperature used in this experiment (30, 31). The data in the absence of GroES can be fitted optimally to a four-phase mechanism (30); however, the presence of GroES introduces an extra quench phase (see fitting residuals in Fig. 2, B and C). Hence, the full description of the ATP-driven system in the absence of GroES can be written as in Scheme 1,

\[
T \leftrightarrow R_1 \leftrightarrow R_2 \leftrightarrow R_3 \leftrightarrow R_4
\]

SCHEME 1

and the ATP- and GroES-driven system is described by Scheme 2.

\[
T \leftrightarrow R_1 \leftrightarrow R_2 \leftrightarrow R_3 \leftrightarrow R_3^* \leftrightarrow R_4
\]

SCHEME 2

Because the final kinetic phase observed in the presence and absence of GroES is a fluorescence enhancement, we have placed the additional kinetic phase observed in the presence of GroES after $R_3$ giving rise to the $R_3^*$ intermediate species.

To determine the point along this pathway at which GroES associates we examined the influence of GroES on each of the kinetic phases. The rate of the fast $T \rightarrow R$ transition (observable here at 150 $\mu$M ATP) was unperturbed by the presence of GroES (Fig. 2D). In addition, the ATP dependence of the first order observed rate constant of the first fluorescence quench phase (the $R_1 \rightarrow R_2$ transition) was also unaffected by the presence of GroES (Fig. 3B). This was also the case in the presence of GroES (Fig. 3B and indicated by the dashed line in Fig. 3A). However, upon examining the amplitude of the $R_1 \rightarrow R_2$ phase as a function of the concentration of ATP, it can be seen that it was clearly influenced by the presence of the co-protein (Fig. 3C). The ATP dependence of the amplitude of this phase fits optimally to the Hill equation with an $K_{1/2}$ of 13.0 ($\pm$0.36) $\mu$M and a Hill coefficient of 2.49 ($\pm$0.14) in the absence of GroES and $K_{1/2}$ of 6.85 ($\pm$0.10) $\mu$M and a Hill coefficient of 2.28 ($\pm$0.06) in the presence of GroES. This effect can only be explained by the binding of GroES to the $R_2$ state, thereby increasing the apparent binding affinity for ATP and shifting the amplitude curve to the left. Thus the above, qualitative analysis leads to the tentative conclusion that GroES interacts with the $R_2$ state.

From these data we can thus infer that the reaction scheme (Reaction 1) in the presence of ATP and GroES is as follows.

\[
\text{FIGURE 3. The effect of GroES on the } R_1 \rightarrow R_2 \text{ rearrangement. The rate constants for the first quench phase } R_1 \rightarrow R_2 \text{ were recorded when 0.5 $\mu$M GroEL (oligomers) was challenged with a series of ATP concentrations (graph A). The experiment was performed in the absence of GroES (open circles) and in the presence of 1 $\mu$M GroES oligomers (filled circles). The data show that GroES had no effect on the rate of the transition. Graph B shows the effect of ADP on the same rate constant at a concentration of 1.5 mM ATP. At saturation, ADP abolished the second sigmoidal phase seen in A whether GroES was present (open circles) or not (filled circles). The dotted line in A represents the asymptotic approach to the ADP-inhibited rate and illustrates the abolition of the second phase. Shown in C are the amplitude data for this first quench phase (} R_1 \rightarrow R_2 \text{) with and without GroES and in the absence of ADP. In the presence of the co-protein the amplitude curve is clearly shifted to the left showing that the co-protein must interact with } R_2.\]

Encapsulation Mechanism of GroEL
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The observed rate constants for the \( R_2 \rightarrow R_3 \) (graph A), the \( R_3 \rightarrow R_3^* \) (graph B), and the \( R_3^* \rightarrow R_4 \) (graph C) transitions of 0.5 \( \mu M \) GroEL oligomers in the presence of 1 \( \mu M \) GroES are shown as a function of the concentration of ATP. For graphs A and B the data were fitted to the Hill equation and gave values for \( n_h \) of 2.5 \((\pm 0.9)\) and 3.4 \((\pm 0.7)\), respectively, and for \( K_{1/2} \), the values were 334 \((\pm 52)\) and 276 \((\pm 1.7)\) \( \mu M \). For the \( R_3^* \rightarrow R_4 \) transition (graph C) the rate constant for this final phase did not appear to be sensitive to the concentration of the nucleotide. However, the amplitude of the fluorescence enhancement associated with the \( R_3^* \rightarrow R_4 \) transition (shown in graph D) was saturable with ATP. When fitted to the Hill equation values for \( n_h \) and \( K_{1/2} \) were 1.92 \((\pm 0.25)\) and 136 \((\pm 9)\) \( \mu M \), respectively. Note that at low concentrations of ATP \((<50 \mu M)\) the magnitude of the rate constants in B and C are similar, thus introducing large errors into the fitting parameters at these concentrations.

\[
T \cdot ATP \rightleftharpoons R_1 \cdot ATP \rightleftharpoons R_2 \cdot ATP \rightleftharpoons R_3 \cdot ATP \cdot GroES
\]

\[
\uparrow
\]

\[
R_1 \cdot ATP \cdot GroES
\]

\[
\uparrow
\]

\[
R_2 \cdot ATP \cdot GroES
\]

\[
\uparrow
\]

\[
R_3 \cdot ATP \cdot GroES
\]

REACTION 1

This interpretation assumes that if GroES binds to a particular state it will affect its optical or kinetic properties. It is possible that GroES binds before the GroEL conformation acquires the \( R_2 \) conformation, but that the binding is optically and kinetically silent. However, with such a large protein cofactor that is known to have very large effects on the conformation of GroEL, this appears unlikely.

Dependence of Observed Rate Constants on the Concentration of ATP—We next assessed the effect of the concentration of ATP on the observed rate constants for the nucleotide-induced rearrangements (Fig. 4), so that we could compare the responses of the system to ATP in the presence and absence of GroES. As described in the previous section, the first enhancement \((T \rightarrow R_1)\) is entirely unaffected by the presence of GroES, and the first quench phase \((R_1 \rightarrow R_2)\) is affected only in its amplitude, and there is no effect on rate. The second quench phase, designated \((R_2 \rightarrow R_3)\), peaked at a rate constant of \(-14 \text{ s}^{-1}\) in the presence of GroES (Fig. 4A); compared with \(-6 \text{ s}^{-1}\) for the second quench in the absence of GroES (30). The third quench phase \((R_3 \rightarrow R_3^*)\) did not occur at all in the absence of GroES and had a maximum first-order rate constant of \(-1 \text{ s}^{-1}\) at saturating concentrations of ATP (Fig. 4B). The final kinetic phase, a fluorescence enhancement phase \((R_3^* \rightarrow R_4)\), showed no variation in rate as ATP concentration increased, being a constant \(0.26 \text{ s}^{-1}\) in the presence of GroES (Fig. 4C). This compares with a rate constant of \(-0.6 \text{ s}^{-1}\) for the final enhancement phase in the absence of GroES. However, the amplitude of the \(R_3^* \rightarrow R_4\) phase increased with the concentration of ATP and showed half-saturation at a concentration of \(-140 \mu M\) (Fig. 4D).

Progressive Tightening of the GroEL-GroES Complex after the Initial Interaction—By maintaining a constant concentration of ATP (1 \( \mu M \)) and varying the concentration of GroES we were able to use phase-amplitude analysis to make an estimate of the GroES binding affinity as the GroEL molecule progresses through its ATP-driven rearrangements (Fig. 5). It can be seen that the amplitude of the second quench phase \((R_2 \rightarrow R_3)\) is enhanced as the concentration of GroES is increased from sub-stoichiometric to super-stoichiometric levels (Fig. 5A). This phase of the reaction represents the process in Reaction 2.

\[
R_2 \cdot ATP \rightleftharpoons R_2 \cdot ATP \cdot GroES \rightleftharpoons R_3 \cdot ATP \cdot GroES
\]

REACTION 2

If the first binding step is represented by the following dissociation constant (Equation 1),

\[
K_{d(R_2 \cdot GroES)} = \frac{[R_2 \cdot ATP] \cdot [GroES]}{[R_2 \cdot ATP \cdot GroES]} \quad \text{(Eq. 2)}
\]

and the second by the unimolecular equilibrium constant \(K_{(2,3)}\) (Equation 2), where,

\[
K_{(2,3)} = \frac{[R_2 \cdot ATP \cdot GroES]}{[R_2 \cdot ATP]} \quad \text{(Eq. 3)}
\]
Encapsulation Mechanism of GroEL

The Dynamics of a Bound Polypeptide: the Experimental System—The binding of ATP weakens the affinity of GroEL for polypeptide (13, 15, 32, 41); therefore, the conundrum arises as to why the initial binding of ATP to a GroEL-protein substrate complex does not cause unfolded polypeptide to be released into the bulk solution before GroES has associated, especially because the binding sites for the two ligands overlap on the GroEL apical domains. Is GroES association so rapid as to prevent significant loss of polypeptide, or has GroEL evolved a mechanism to ensure efficient capture of the polypeptide into the central cavity beneath GroES? One way to address this question is to determine at which point along the ATP-induced allosteric pathway the polypeptide “senses” the conformational rearrangements in GroEL. Complications in the kinetics caused by the refolding of the protein substrate were avoided by using \( \alpha \)-lactalbumin, which had been reduced and carboxymethylated (CM-LA). The removal of Ca\(^{2+} \) and the reduction of the disulfide bonds in \( \alpha \)-lactalbumin resulted in the protein fully unfolding, and its interaction with GroEL has been well characterized (33–36). Modification of the sulfhydryl groups of \( \alpha \)-lactalbumin by carboxymethylation maintains the polypeptide in a non-native conformation. The polypeptide was also labeled using dansyl chloride, which modifies primary amine groups, to monitor any changes to its environment. Labeling conditions were chosen such that the \( \alpha \)-amino group at the N terminus would be the most reactive amine, and the final ratio of label:polypeptide was, on average, 1:1. The effect of bound polypeptide on ATP-induced structural rearrangements in then the apparent overall binding affinity reported by the amplitude plot \( (K_{d1ES(app)}) \) is given by Equation 3.

\[
K_{d1ES(app)} = \frac{K_{dR1ES}}{(1 + K_{d2,3^*})}
\]  
(Eq. 4)

The amplitude data in Fig. 5A can be fitted to the tight-binding equation with \( K_{d1ES(app)} = 190 \) (±80) nM, although it must be stated that these data do not accurately resolve this binding affinity, a fact reflected in the degree of error.

A similar kind of analysis can be performed for the last two kinetic phases, \( R_3 \rightarrow R_3^* \) and \( R_3^* \rightarrow R_4 \). The fluorescence amplitude of the \( R_3 \rightarrow R_3^* \) phase, again at a fixed ATP concentration of 1 mM, also increases with increasing GroES concentration and can be fitted to determine the apparent affinity of GroES at this stage of the reaction. Although it is difficult to resolve accurate data at low concentrations of GroES, the data in Fig. 5B fit optimally to the tight-binding equation with \( K_{d2,3ES(app)} = 15.0 \) (±14.1) nM, ~10-fold tighter than \( K_{d1ES(app)} \). Although the effective binding affinity is poorly determined, we take these data as evidence that there is a tightening of the GroES interaction as the GroEL molecule progresses from the \( R_3 \) to the \( R_3^* \) conformation.

The presence of increasing concentrations of GroES also enhanced the amplitude of the \( R_3^* \rightarrow R_4 \) phase (Fig. 5C). The data fit optimally to the tight-binding equation with a \( K_{d1ES(app)} = 4.39 \) (±2.86) nM. This probably represents a further tightening of the GroEL-GroES interaction as the reaction progresses from \( R_3^* \) to \( R_4 \), although the errors in fitting the amplitudes of multieponential are considerable.

**FIGURE 5.** Measured binding affinities for the GroEL-GroES interaction. A mixture of GroES and ATP (3 mM) was rapidly mixed with a solution of GroEL (0.5 \( \mu \text{m} \) oligomer) and the fluorescence intensity emitted from Trp-485 was recorded (as exemplified in Fig. 2). The reaction was repeated at a series of concentrations of GroES, and the amplitudes for the second quench \( (R_3 \rightarrow R_3^*) \), the third quench \( (R_3^* \rightarrow R_4^*) \), and the final enhancement \( (R_4^* \rightarrow R_4) \) are shown as a function of the GroES concentration in A–C, respectively. The data have been fitted to the quadratic tight ligand binding equation shown under “Experimental Procedures,” and the apparent binding constants were 190 (±80) nM, 15.0 (±14.1) nM, and 4.39 (±2.86) nM, respectively. Note that at this concentration of ATP the magnitude of the rate constants of the various kinetic phases are sufficiently well separated to keep errors to a minimum.
Encapsulation Mechanism of GroEL

GroEL was then assessed using the intrinsic fluorescence of Trp-485-GroEL. However, in this case, to avoid contamination of the fluorescence signal by the presence of the four native tryptophan residues in α-lactalbumin, the CM-LA was treated with Koshland’s reagent (HNBB), which forms a non-fluorescent adduct with the indole group so that substrate fluorescence does not interfere with the indole signal from Trp-485 in GroEL (37).

Association of Unfolded α-Lactalbumin and GroEL—The kinetics of association of GroEL with either dansyl-CM-LA or HNBB-treated CM-LA were investigated by monitoring either the fluorescence of the dansyl-CM-LA or the intrinsic fluorescence of Trp-485 in GroEL, respectively. Association of the protein and GroEL did not cause any change in the fluorescence intensity of the Trp-485 probe in GroEL, suggesting that the binding of the dansyl-CM-LA does not result in any significant structural movements in GroEL (data not shown). Association of CM-LA and GroEL monitored by dansyl fluorescence revealed a large fluorescence enhancement, which could be fitted optimally to three exponentials (Fig. 6A). Upon varying the concentration of GroEL the fastest observed rate constant varied linearly suggesting that it represents the formation of the collision complex and is under pseudo-first order conditions (Fig. 6B). The data in Fig. 6B were fitted to Equation 5.

\[ k_{\text{obs}} = k_{\text{on}}[\text{GroEL}] + k_{\text{off}} \]

(Eq. 5)

The fit to the data gave values, \( k_{\text{on}} = 2.56 \times 10^6 (\pm 0.46) \text{ M}^{-1} \text{s}^{-1} \) and \( k_{\text{off}} = 8.1 (\pm 3.77) \text{ s}^{-1} \). The apparent \( K_d \) of CM-LA, calculated from \( k_{\text{off}}/k_{\text{on}} \), was \( \sim 3 \text{ mM} \). A plot of the amplitudes of these phases as a function of the GroEL concentration (data not shown) can also be fitted to a binding hyperbola with a \( K_d \) of \( \sim 2 \mu \text{M} \), which is in excellent agreement with that obtained from the rate constants. The presence of more than one kinetic phase of binding is likely to reflect some heterogeneity in the conformational populations as well as some degree of conformational rearrangement on the binding surface of the chaperonin; however, all analyses indicated that CM-LA binds with micromolar affinity.

Polypeptide Is Ejected from Its Binding Site after the Binding of GroES—To determine when a bound polypeptide senses the nucleotide-induced conformational rearrangements in GroEL a binary complex of dansyl-CM-LA-GroEL was formed at a 0.5:1 ratio and rapidly mixed with ATP, in the absence and presence of GroES, and changes in the fluorescence intensity of the dansyl group attached to the CM-LA were monitored. The rapid mixing of the wtGroEL-dansyl-CM-LA binary complex with 1 mM ATP alone produced a biphasic decrease in dansyl fluorescence intensity that could be fitted to a double exponential decay with a rate constant for the major phase of \( \sim 0.7 \text{ s}^{-1} \) and a minor, low amplitude fast phase with a rate constant of \( 9 \text{ s}^{-1} \) as shown in Fig. 7A. Upon mixing the same binary complex with 1 mM ATP and GroES, a biphasic kinetic profile was observed, although the amplitude of the faster phase now dominated the signal; the parameters being \( k_{\text{obs,1}} \sim 11 \text{ s}^{-1} \) and \( k_{\text{obs,2}} \sim 0.2 \text{ s}^{-1} \), with relative amplitudes of 0.32 and 0.1, respectively (Fig. 7A). The faster of these two processes corresponds well to the saturating rate constant observed for the \( R_2 \) to \( R_3 \) step.
Encapsulation Mechanism of GroEL

The binding and hydrolysis of ATP by GroEL drives the mechanism of assisted protein folding. ATP binding is cooperative such that the subunits within a heptameric ring are coupled in a positive manner and behave concertedly, whereas negative cooperativity between the rings leads to asymmetry (18, 21). The large structural motions induced by the binding of ATP are necessary to trigger association with the GroES co-protein and eject substrate polypeptide into the central cavity (7, 8, 25, 40). The data presented here attempts to elucidate the effect of GroES on the ATP-induced structural rearrangements in GroEL and to determine at which point the protein substrate is affected by these conformational changes.

In this study, the engineered single tryptophan at position 485 within GroEL was used to probe ATP-induced conformational changes in the presence of GroES. The resulting fluorescence changes upon mixing GroEL with GroES and ATP were remarkably similar to those observed in the absence of GroES (30). A very rapid fluorescence enhancement is followed by multiple fluorescence quenching phases before a final slow enhancement. The principle difference between transients in the absence and presence of GroES is the addition of an extra enhancement. The principle difference between transients in the absence and presence of GroES is the addition of an extra enhancement. The principle difference between transients in the absence and presence of GroES is the addition of an extra enhancement. The principle difference between transients in the absence and presence of GroES is the addition of an extra enhancement.

The rate constants of the phases between $T$ and $R_3$ (see Scheme 1 and Fig. 8) were unaltered, but the new phase clearly preceded the slow fluorescence enhancement ($R_3$ to $R_4$ in optimally to a double exponential decay with parameters $k_{obs,1} = 12.6 \pm (0.52) \text{ s}^{-1}$ and $k_{obs,2} = 0.8 \pm (0.1) \text{ s}^{-1}$ with fractional amplitudes of 0.68 and 0.32, respectively. C, the ATP dependence of the two kinetic phases observed in the top panel were determined ($k_{obs,1}$, main panel; $k_{obs,2}$, inset). The variation of $k_{obs,2}$ with ATP can be fitted to the Hill equation with parameters $k_i = 11.3 \pm (0.8) \text{ s}^{-1}$, $K_{1/2} = 223 \pm (33) \mu M$, and Hill constant $= 1.25 \pm (0.23)$. Experimental conditions were as described in A.

To confirm that dansyl-CM-LA was sensitive to the conformational changes in GroEL occurring in the $R_2$ to $R_3$ transition we determined the ATP dependence of the rate constants of the two kinetic phases observed when a GroEL:danysl-CM-LA binary complex was rapidly mixed with GroES and ATP (Fig. 7C). The variation of the faster phase ($k_{obs,1}$) with ATP concentration can be fitted optimally to a Hill equation with parameters $k_i = 11.3 \pm (0.8) \text{ s}^{-1}$, $K_{1/2} = 223 \pm (33) \mu M$, and a Hill constant $= 1.25 \pm (0.23)$. These values are very similar to those observed for the $R_2 \rightarrow R_3$ conformational transition in GroEL shown in Fig. 4A. The ATP dependence of the second, slower kinetic phase was also determined (Fig. 7C, inset). This observed rate constant for this phase is similar to that seen in the $R_3^* \rightarrow R_4$ transition (see Fig. 4C) and also varied only slightly with ATP concentration.

**DISCUSSION**

The binding and hydrolysis of ATP by GroEL drives the mechanism of assisted protein folding. ATP binding is cooperative such that the subunits within a heptameric ring are coupled in a positive manner and behave concertedly, whereas negative cooperativity between the rings leads to asymmetry (18, 21). The large structural motions induced by the binding of ATP are necessary to trigger association with the GroES co-protein and eject substrate polypeptide into the central cavity (7, 8, 25, 40). The data presented here attempts to elucidate the effect of GroES on the ATP-induced structural rearrangements in GroEL and to determine at which point the protein substrate is affected by these conformational changes.

In this study, the engineered single tryptophan at position 485 within GroEL was used to probe ATP-induced conformational changes in the presence of GroES. The resulting fluorescence changes upon mixing GroEL with GroES and ATP were remarkably similar to those observed in the absence of GroES (30). A very rapid fluorescence enhancement is followed by multiple fluorescence quenching phases before a final slow enhancement. The principle difference between transients in the absence and presence of GroES is the addition of an extra quench phase when the co-protein is present.

The rate constants of the phases between $T$ and $R_3$ (see Scheme 1 and Fig. 8) were unaltered, but the new phase clearly preceded the slow fluorescence enhancement ($R_3$ to $R_4$ in optimally to a double exponential decay with parameters $k_{obs,1} = 12.6 \pm (0.52) \text{ s}^{-1}$ and $k_{obs,2} = 0.8 \pm (0.1) \text{ s}^{-1}$ with fractional amplitudes of 0.68 and 0.32, respectively. C, the ATP dependence of the two kinetic phases observed in the top panel were determined ($k_{obs,1}$, main panel; $k_{obs,2}$, inset). The variation of $k_{obs,2}$ with ATP can be fitted to the Hill equation with parameters $k_i = 11.3 \pm (0.8) \text{ s}^{-1}$, $K_{1/2} = 223 \pm (33) \mu M$, and Hill constant $= 1.25 \pm (0.23)$. Experimental conditions were as described in A.

**FIGURE 7.** ATP-induced dissociation of a fluorescently labeled, unfolded protein substrate from GroEL. A, CM-LA (2 μM), covalently labeled with dansyl chloride was mixed with GroEL (4 μM) to form a protein-protein complex. This complex was challenged in a stopped-flow fluorometer with ATP (2 mM) or a mixture of ATP and GroES (8 μM), and the fluorescence was recorded as described under “Experimental Procedures” and the legend to Fig. 5. The dissociation process is biphasic with observed rate constants of $k_{obs,1} = 8.6$ and $k_{obs,2} = 0.7 \text{ s}^{-1}$ in the absence of GroES (relative fractional amplitudes, 0.22 and 0.78) and 11.3 and 0.2 s$^{-1}$ in the presence of GroES (relative fractional amplitudes, 0.76 and 0.26). The first 1 s of the progress curve is shown, but the double exponentials were fitted over a time sweep of 4 s. Concentrations given are before mixing. B, the experiment described in the top panel was repeated using SR1 instead of GroEL. The fluorescence quench phases observed upon rapidly mixing with ATP and GroES were fitted repeating the experiment using the engineered single-ring version of GroEL known as SR1 (7). The absence of the second ring has been shown not to alter the kinetics and pathway of the ATP-induced conformational rearrangements within a GroEL ring (38, 39). The transient change in dansyl fluorescence intensity when a dansyl-CM-LA:SR1 binary complex was rapidly mixed with ATP, in the presence of GroES, was identical to that observed with the double ring (Fig. 7B) thus showing that the fluorescence changes from dansyl-CM-LA are not due to effects in the opposite ring.

To confirm that dansyl-CM-LA was sensitive to the conformational changes in GroEL occurring in the $R_2$ to $R_3$ transition we determined the ATP dependence of the rate constants of the two kinetic phases observed when a GroEL:dansyl-CM-LA binary complex was rapidly mixed with GroES and ATP (Fig. 7C). The variation of the faster phase ($k_{obs,1}$) with ATP concentration can be fitted optimally to a Hill equation with parameters $k_i = 11.3 \pm (0.8) \text{ s}^{-1}$, $K_{1/2} = 223 \pm (33) \mu M$, and a Hill constant $= 1.25 \pm (0.23)$. These values are very similar to those observed for the $R_2 \rightarrow R_3$ conformational transition in GroEL shown in Fig. 4A. The ATP dependence of the second, slower kinetic phase was also determined (Fig. 7C, inset). This observed rate constant for this phase is similar to that seen in the $R_3^* \rightarrow R_4$ transition (see Fig. 4C) and also varied only slightly with ATP concentration.
Encapsulation Mechanism of GroEL

![Diagram](image)

Scheme 1); therefore, we postulated the presence of a further conformational intermediate between $R_3$ and $R_4$, which we have termed $R_3^*$. The kinetic phase corresponding to the $T$ to $R_1$ transition was unaffected by the presence of GroES, indicating that the co-protein was not bound at this point in the reaction (see the summary depicted in Fig. 8). The rate constant for the next phase ($R_1$ to $R_2$) was also unperturbed by the presence of GroES. However, the dependence of the amplitude on ATP concentration revealed that the binding affinity of ATP was enhanced in the presence of GroES (Fig. 3B). This can only be explained by the binding of GroES to the $R_2$ species and thus the result identifies the point at which GroES associated with GroEL. Subsequent transitions in the pathway led to a progressive tightening of the GroEL-GroES interaction (Fig. 5).

The binding of ATP to GroEL reduces the affinity of the chaperonin for bound polypeptide (13, 15, 32, 41) leading to a tendency to release the polypeptide into bulk solution. However, when GroES is present in the reaction mixing a GroEL-polypeptide binary complex with GroES and ATP results in the efficient encapsulation of the polypeptide within the central cavity of the GroEL ring beneath GroES (7, 8). In these circumstances the protein substrate is not bound to the cavity walls and is able to fold unhindered by contacts with the chaperonin that would otherwise retard the folding process. In addition, it has been demonstrated that GroES and the substrate protein have overlapping, if not identical, binding sites on the seven apical domains of GroEL (26–28). This arrangement poses a problem if the protein substrate has to be released to vacate the binding sites for the incoming GroES molecule. If substrate is ejected before association with the co-protein, what stops it diffusing into the bulk solvent?

To address this question we have determined at what stage along the pathway of ATP-induced conformational changes in GroEL the structural rearrangements are "sensed" by the bound polypeptide substrate. Here we have used a model unfolded polypeptide, reduced carboxymethyl-α-lactalbumin, which does not refold in bulk solution, thus avoiding complications due to signal changes as the protein refolds. Additionally, although CM-LA forms a stable binary complex with GroEL, it does not bind so tightly as to put a high load on the GroEL complex, thus enabling the earliest point at which the polypeptide is affected by the structural motions in GroEL to be detected. Indeed a relatively tight binding protein substrate such as mitochondrial malate dehydrogenase or rhodanese places a load on

FIGURE 8. Schematic representation of ATP-induced rearrangements in the GroEL-GroES system. After formation of the initial collision complex, $T$:ATP, there is a rapid and cooperative rearrangement to $R_1$:ATP, which is accompanied by an enhancement of fluorescence emitted by tryptophan 485. This is followed by a conformational change that renders the apical domains receptive to GroES (complex $R_2$:ATP). This rearrangement is not sensed by the bound CM-La protein substrate. In the presence of GroES there is then a rapid formation of the $R_2$:ATP:ES complex, which represents the encapsulation step, followed by a conformational shift to the $R_2$:ATP:ES complex, which is strongly communicated to the protein substrate within the enclosed cavity, the fluorescence signal of which more resembles the free substrate. There is a further step ($R_2$:ATP:ES to $R_3$:ATP:ES) that is also sensed by the protein substrate within the cavity; this may be to a more weakly bound state or may represent displacement of a more tightly bound sub-population of protein substrate molecules. In the absence of GroES, the $R_2$:ATP to $R_3$:ATP and $R_4$:ATP steps are sensed by the bound substrate and occur at slightly slower rates. All rate constants represent the behavior of the system at 1 mM ATP.

Our data show that the bound substrate senses nothing in terms of environment change before the binding of the co-protein, i.e. GroES binds to the $R_3$ conformation, but there was no displacement of the labeled substrate until the shift from the $R_2$ to the $R_3$ state in the GroEL-GroES complex (Fig. 8). This shift in conformation leads to a tightening of the GroES interaction presumably owing to the vacation of additional binding surface as the substrate protein is displaced from its binding site. The fact that the two distinct kinetic phases were observed as the polypeptide was displaced may indicate that the polypeptide is partially released during the first, faster phase ($R_2 \rightarrow R_3$) before being fully displaced into the cavity at the slower observed rate concomitant with the $R_3^* \rightarrow R_4$ transition or that there are two conformational ensembles of bound polypeptide with different kinetics of dissociation (Fig. 7C). However, this order of events, in which GroES binds before the ejection of the substrate polypeptide is initiated, results in all of the polypeptide being efficiently captured in the central cavity beneath GroES.

It has been observed previously by a combination of structural studies and site-directed mutagenesis that the binding sites of an unfolded polypeptide and the mobile loop of GroES overlap to a large extent (11, 26, 28). Residues involved in bind-
ing the polypeptide cluster between β-strands 6 and 7 (residues Tyr-199, Ser-201, Tyr-203, and Phe-204); helix H (Leu-234 and Leu-237); and helix I (Leu-259, Val-263, and Val-264), whereas in the GroEL-(ADP)2-GroES structure helices H and I move to the top of the subunit to provide a binding site for the GroES mobile loop. In addition, polypeptide binding is known to be multivalent, occupying several binding sites within a heptameric ring (44). It may be that during the conformational rearrangements triggered by the binding of ATP, the intermediate R2 has elevated the apical domains sufficiently to permit the relatively weak association of GroES to its binding sites on Helices H and I, but without the twisting motion associated with occlusion of the polypeptide binding sites (11, 27).

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