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The affinity of the GroEL/GroES complex for peptides under conditions of protein folding

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Abstract The affinity of four short peptides for the *Escherichia coli* molecular chaperone GroEL was studied in the presence of the co-chaperone GroES and nucleotides. Our data show that binding of GroES to one ring enhances the interaction of the peptides with the opposite GroEL ring, a finding that was related to the structural readjustments in GroEL following GroES binding. We further report that the GroEL/GroES complex has a high affinity for peptides during ATP hydrolysis when protein substrates would undergo repeated cycles of assisted folding. Although we could not determine at which step(s) during the cycle our peptides interacted with GroEL, we propose that successive state changes in GroEL during ATP hydrolysis may create high affinity complexes and ensure maximum efficiency of the chaperone machinery under conditions of protein folding.

Key words: GroEL; GroES; Nucleotide; Peptide; Cycle; Binding affinity

1. Introduction

The *Escherichia coli* molecular chaperone GroEL and its co-chaperone GroES are the most widely studied members of the molecular chaperone family of proteins whose function is to assist the folding and refolding of substrate proteins [1–3]. The combination of the GroEL/GroES/ADP_7 crystal structure [4] and numerous elegant physical studies using GroEL mutants [5–8] now provides convincing evidence as to how the interaction between GroEL, GroES and nucleotides leads to the controlled cyclical sequestration of substrate protein folding intermediates. This complex reaction cycle of protein folding has been described as a two-stroke folding machine [3,9] and is comprised of several steps (for a recent review see [2]). Briefly, the initial recognition and binding of a non-native protein at a GroES-free ring is followed by the release of the bound polypeptide into the central cavity which is formed through the binding of GroES and ATP at the same ring. The protein substrate is allowed to equilibrate inside this protected environment (sometimes known as Anfinsen's cage) for a fixed amount of time whilst ATP is being hydrolysed to ADP ($t_{1/2}$ 6–8 s) [6,10,11]. A further step of ATP binding at the opposite or *trans* ring leads to the discharge of GroES, ADP and the substrate which may now be in a form that is disposed towards productive folding. In vitro studies with proteins suggest that the nucleotide-free ring with GroES and ADP bound in *trans* represents the acceptor state for substrates [4,5,12]. During its reaction cycle, the chaperone undergoes several combinations of GroES, ATP and ADP bound to its two rings, and it is therefore likely that a non-native protein may encounter, and possibly bind to, all or some of these states with varying strength. The actual substrate affinity of the GroEL ring in *trans* to GroES and its occupancy with nucleotides during in vivo folding conditions remain somewhat ill-defined at present.

For most protein substrates, reaching a native-like state that is no longer recognised by the chaperonin involves many iterative cycles of rebinding and release [13–16]. These structural rearrangements result in successive changes in the affinity of these protein substrates for GroEL and make it difficult to assess the affinity of the GroEL/GroES folding machinery during protein folding conditions, i.e. during cycles of ATP hydrolysis and repeated substrate release. Using peptide substrates as mimetics of non-native proteins has the advantage that this approach circumvents complications through kinetic competition between substrate folding and binding. Consequently, the affinity of the GroEL/GroES machinery under protein folding conditions may be studied more readily. In our previous efforts to describe the phenomenon of molecular recognition and substrate binding by GroEL, we have employed short peptides and looked at the effects of primary and secondary structure on binding by GroEL [17,18]. Since in the cellular environment, GroEL functions in synergy with its co-chaperone GroES and in the presence of nucleotides, we report in this study the results of experiments designed to describe the affinity of the complete GroEL/GroES machinery. The NON-AMPH series of peptides used in this study was originally designed to probe the importance of non-amphiphilic secondary structure to GroEL molecular recognition and substrate binding, and comprises three peptides (sequence: ALYKIKKIKLLESK-ε-dansyl) where one was N-terminally modified with an α-helix stabilising template (NON-$AMPH^+$), one was modified with a non-stabilising template (NON-$AMPH^-$), and one was left unmodified (NON-$AMPH^R$) [17]. These three fluorescent peptides and the parent peptide B_{amph} (sequence: dansyl-PLYKKIIKKLLES) were shown previously to display strong affinities for GroEL with apparent dissociation constants in the nM to μM region (cf. [17,18] and Fig. 1A). In this paper we present the results of experiments designed to describe the effects of GroES and nucleotides such as ATP and ADP on the affinity of GroEL for peptides.

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2. Materials and methods

2.1. Protein and peptide preparation

GroEL and GroES were purified from a recombinant strain of *E. coli* according to previously published methods [17,19]. The protein concentrations given always refer to the oligomer concentration. The peptides (NON-AMPH$^+$, NON-AMPH$^-$, NON-AMPH^R and B_{amph}) were prepared as described previously [17,18].

2.2. Fluorescence binding assays

Fluorescence GroEL–peptide equilibrium binding assays were performed as described previously [17]. Briefly, pre-equilibrated peptide solutions (1.0 μM) were titrated with GroEL or equimolar amounts of GroEL and GroES (in 50 mM Tris–HCl pH 7.6, 2 mM dithiothreitol) until the fluorescence intensity enhancement appeared to be saturating. Given concentrations refer to the GroEL concentration. At each addition, the dansyl fluorescence emission intensities at 500 nm (excitation at 350 nm), corrected for protein background, were tabulated and the intensity of free peptide was subtracted in order to give the fluorescence intensity enhancement, ΔI_{500}. ΔI_{500} data were then analysed as a function of the GroEL concentration using a model for binding in which a given peptide was assumed to be able to interact with an unspecified number of independent binding sites on the GroEL homo-oligomer [18]:

$$\Delta I_{500} = (\Delta\phi[P]_t[G]_t)/(K_d + [G]_t) \quad (1)$$

where $[P]_t$ is the total peptide concentration (1 μM), $[G]_t$ the total GroEL concentration, K_d the apparent dissociation constant, and $\Delta\phi$ a term deriving from the fluorescence quantum yield enhancement upon peptide binding to GroEL.

Binding assays were performed in buffer A (50 mM Tris–HCl pH 7.6, 2 mM dithiothreitol), buffer B (buffer A, 10 mM KCl, 10 mM $MgCl_2$), ADP buffer (buffer B, 8 mM ADP), and ATP buffer (buffer B, 8 mM ATP) with either GroEL or equimolar amounts of GroEL and GroES at 20°C. A concentration of 8 mM ATP was chosen to ensure on-going hydrolysis over the time course of the titration experiment (approximately 1 h), and to minimise product inhibition by ADP.

3. Results

3.1. Binding in the absence of GroES

Fig. 1A shows the dissociation constants of interaction of the four peptides with GroEL. Strongest binding was found under conditions of low ionic strength in our standard assay buffer A. Consistent with previous results [17], the affinity of the complex was substantially weakened in buffer B where magnesium and potassium ions were included to obtain conditions allowing ATP hydrolysis for later experiments [20,21]. This decrease in affinity is likely to be due to shielding of favourable electrostatic attractions between the negatively charged GroEL protein [22] and the positively charged peptides by salts [17].

The presence of ATP was found to lower the affinity of GroEL for the peptides significantly, a finding that is consistent with the literature [23–25]. Nucleotide binding to GroEL occurs in a co-operative manner involving one ring at a time [26–28], and is known to induce conformational changes [4,5,24,29–31] where the nucleotide-free ring (T-state) and the nucleotide-bound ring (R-state) display a high and low affinity for substrate proteins respectively [26,27]. The affinity of the GroEL/ADP complex for the peptides (static situation) was found to be enhanced relative to the dynamic situation under conditions of ATP hydrolysis. This finding is consistent with the fact that GroEL displays weaker binding for ADP relative to ATP [32,33], and that higher concentrations of ADP are required for the transition of high to low affinity complexes [12].

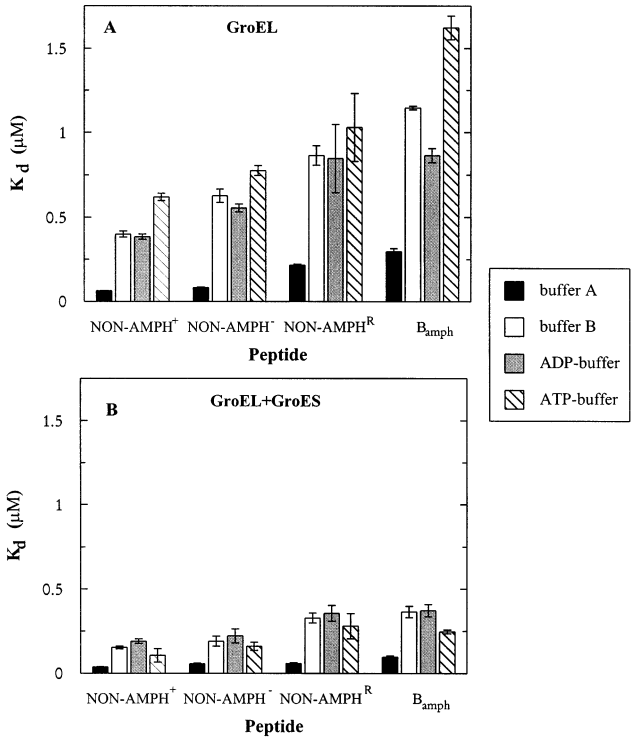


Fig. 1. A: Column chart of dissociation constants (K_d) on interaction of peptides with GroEL (μM). B: Column chart of dissociation constants (K_d) on interaction of peptides with GroEL:GroES = 1:1 (μM). Error margins in both panels were derived from the S.E.M.s of the binding isotherms.

3.2. Binding in the presence of GroES

Fig. 2 shows representative isotherms for binding of the NON-AMPH$^+$ peptide under conditions where both GroEL and GroES were present in the reaction mixture. The resulting dissociation constants for all peptides are illustrated in Fig. 1B. The presence of a twofold excess of GroES over GroEL was found not to alter peptide binding to GroEL by more than the experimental error (buffer A, not shown). This confirmed that the peptides did not interact strongly with free GroES [17].

Fig. 1B shows that the affinity of GroEL/GroES complexes for peptides under conditions of protein folding (dynamic situation, ATP buffer) was raised slightly above the affinities of the static complexes, the nucleotide-free state (buffer B) and the resting complex (ADP buffer), generally thought to be the acceptor state for substrate proteins (cf. complex **1** in Scheme 1) [4,5,12]. This contrasts with results presented in Fig. 1A where ATP was found to weaken the affinity throughout.

4. Discussion

4.1. Binding to GroEL only

The allosteric readjustments that occur as a consequence of the binding of nucleotides to GroEL control the affinity of a GroEL ring for substrate proteins/peptides [12]. Recent cryo-electron microscopy images of GroEL provide insight into the structural changes involved [1,29–31]. Nucleotide binding to one GroEL ring has been shown to disrupt the continuity of the binding surface of this ring (R-state), thereby lowering its accessibility and affinity for polypeptide chains. Slight changes also occur at the opposite T-state ring, which appears verti-

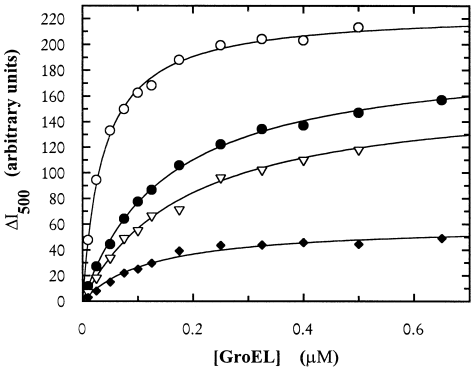


Fig. 2. Representative binding isotherms for binding of the NON-AMPH$^+$ peptide to GroEL/GroES complexes. The fluorescence intensity enhancement ΔI_{500} is shown as a function of the GroEL concentration. The curves through the data sets are fits to the binding model (Eq. 1), the calculated values for the dissociation constants are shown in Fig. 1B. Binding in buffer A (○), buffer B (●), ADP buffer (▽), and ATP buffer (♦).

cally extended but closed by an inward twist in a clockwise rotation. Overall, this leads to a progressive elongation of the structure upon the transition from the nucleotide-free TT-state to the TR-state at low concentrations of nucleotide and the RR-state at high concentrations of nucleotide [12,27,29]. These structural changes may explain the hierarchy of the decrease in affinity shown in Fig. 1A. The fact that the nucleotide-free GroEL ring was found to display the highest affinity for substrates, followed by an ADP-liganded, and then the ATP-bound conformation, has also been reported elsewhere [25,26].

A concern raised with many in vitro studies is the concentration of nucleotide which is generally much lower than what would be expected in vivo. The nucleotide concentrations chosen in this study were physiological with respect to ATP in the cell (8 mM), but unusually high with regards to ADP in *E. coli* (1 mM) [34]. Another concern is the physiological relevance of the allosteric transitions of GroEL that take place in vitro at ATP concentrations much lower than those in *E. coli* [35]. The concentrations of ATP and ADP where the transition from asymmetric to symmetric nucleotide-bound complexes takes place, as well as the possibility that GroES may modulate the occupancy of GroEL with nucleotide, are not yet fully defined [6,8,12,27,36]. It is likely, however, that mostly symmetric complexes were present under our experimental conditions.

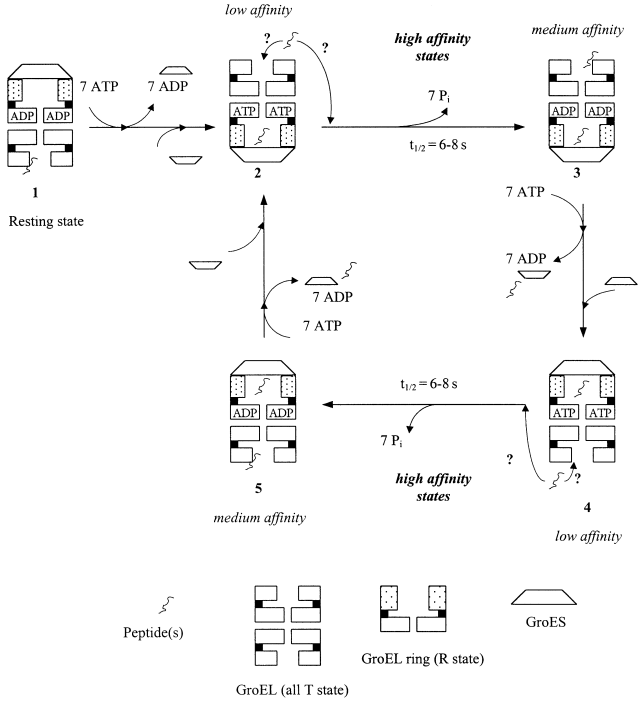
4.2. GroES enhances peptide binding to GroEL

GroEL and GroES are thought not to associate very tightly in the absence of nucleotides [12,32,36,37] (buffer A), although these complexes may form more readily in buffer B for reasons of salt shielding of electrostatic repulsion between the two acidic proteins [22]. Given the fact that the peptide binding sites of GroEL are well known to double up as GroES binding sites [38–41], GroEL/GroES complexes, once formed, are likely to offer only half of the binding regions [17]. This would approximately double the value of the peptides' apparent dissociation constants provided that binding of GroES in *trans* does not affect the affinity of the binding-competent ring. Fig. 1B shows that the binding of peptides to complexes of GroEL and GroES was stronger than in the absence of GroES under the same experimental conditions (Fig. 1A). The effect of GroES to enhance peptide binding agrees with an increase in affinity of GroEL for nucleotides in the presence of the co-chaperonin and vice versa [12,20,32,33,37]. Taken together these findings would appear to suggest that GroES acts as a co-operative organiser by increasing the affinity of the complex not only for nucleotides, but also for substrates at the opposite ring.

Changes in affinity may be explained by the crystal structure of the GroEL/GroES/ADP complex [4]. Binding of the co-factors to one GroEL ring was shown to result in structural readjustments which are compensated for by movements of the *trans* ring where they cause an outward tilt that has been thought responsible for the opposed binding of a second GroES [4]. It seems attractive to speculate that this outward tilt may result in conformational changes in the binding sites which altered, possibly increased, the affinity of GroEL for our peptides when compared to the inward twist and closing of the ring upon ATP binding in the absence of GroES [29].

4.3. Binding to GroEL/GroES/nucleotide complexes

In the presence of ADP, GroEL and GroES associate very tightly with microscopic dissociation constants in the low nM region [12,20,32,33,36,37], forming a static nucleotide-bound complex in which GroES does not exchange during a time course of hours if ATP is not present (complex **1**, Scheme 1) [12,36]. This contrasts with the dynamic situation in ATP



Scheme 1. Schematic model for the reaction cycle of GroEL and GroES, adapted from Horwich and co-workers [4–6] and borrowing from the notation of Sparrer and Buchner [12]. Our results suggest the creation of complexes of high affinity during the slow step of ATP hydrolysis (**2** to **3** and **4** to **5**) where GroEL may undergo up to seven co-operative state changes [26,27]. Peptide may bind with low affinity to the ATP-bound complex (**2** and **4**) [5], but definitely enters the complex before the formation of the ADP form (**3** and **5**). The affinities of complexes **3** and **5** are probably identical to the medium affinity of the resting state **1** (Fig. 1B). Fluorescent peptide inside the cavity is assumed to be spectroscopically indistinguishable from peptide in the bulk solution.

buffer where ternary complexes associate and dissociate rapidly after hydrolysis, and where, every 15 s or so, sites available for peptide binding may switch between the two GroEL rings [3–6,42]. This cycle and possible points of entry for peptide are depicted in Scheme 1. The affinity of the resting complex **1** was determined to be of medium strength (ADP buffer) and is probably identical to the affinity of the transiently occurring complexes **3** and **5**. As to the affinity of the ATP-bound complex (**2** and **4**), a recent study by Rye et al., using the hydrolysis-deficient GroEL mutant D389A, suggested that hydrolysis of ATP must occur before a non-native polypeptide and GroES can bind to the opposite ring [5]. This result has been related to slight differences in cryo-electron microscopy images of the *trans* ring of ATP-bound (**2** and **4**) compared to ADP-bound (**3** and **5**) GroEL/GroES complexes. A slight rotation of the binding sites appears to reduce the accessibility of the binding sites and is probably responsible for the low binding affinity of the ATP-bound complex [5].

The discussion of results obtained during ATP hydrolysis, where available binding sites alternate between both rings (Scheme 1), is more controversial than the static situation in ADP buffer (or using D389A) where peptide binding was probably confined to one ring. Our result of a relatively high affinity complex for peptides under dynamic conditions of ATP hydrolysis is somewhat astonishing, particularly since static ATP-bound and ADP-bound complexes were found to display only low [5] and moderate affinities respectively (Fig. 1B). This appears to contrast with affinity studies in the absence of GroES where ATP was found to lower the affinity (Fig. 1A) and suggests that the interaction of the peptides with GroEL is co-ordinated by GroES. Studies of the reaction cycle show that the conversion of complex **2** to **3** (and **4** to **5**) is the slowest step in the cycle and is regulated by the co-operative hydrolysis of seven ATP molecules with a half-time of 6–8 s [5,6,26,27]. In interpreting our results we would like to put forward the suggestion that this conversion of GroEL/GroES/ATP_7 to GroEL/GroES/ADP_7 complexes leads to co-operative state changes in GroEL and the formation of up to seven transiently occurring intermediates, some or all of which must have a high affinity for peptides. This results in a dynamic complex which displays an affinity greater than that of the ATP-bound complex [5] and slightly greater than that of the ADP-bound state (Fig. 1B). We cannot rule out the possibility that state transitions during the conversion of complex **3** to **4** (and **5** to **2**) may also contribute to the binding as our assay only measures the sum of all interactions. However, taking into account that these conversions take place at the diffusion-controlled limit (compared to the slow steps of hydrolysis), in our view, it is unlikely that these intermediates contribute significantly to the overall affinity.

5. Conclusions

Previous reports on the refolding of protein substrates in the presence of ATP reported a loss of affinity between the chaperone and substrate proteins [12,23–25]. However, under these folding conditions, protein substrates are likely to experience a loss of binding-competent surfaces with each cycle of substrate binding, folding in the cavity, and release. The use of short peptides as mimetics of denatured proteins circumvents this complication of kinetic competition between substrate folding and binding and allows investigation into equilibrium binding during ATP hydrolysis.

Our data, presented in Fig. 1B, provide evidence for a high affinity state of peptide substrate binding of the chaperone machinery during protein folding conditions. By extrapolation, it would appear likely that the same may be true for protein substrates although their affinity may not be studied so readily. We cannot determine exactly at which step(s) during this dynamic cycle our peptides interacted with GroEL, but our data suggest that GroEL may undergo a number of co-operative state changes with high peptide affinity during the slow hydrolysis of the seven GroEL-bound ATP molecules. This result may be intuitively acceptable bearing in mind that many GroEL oligomers undergo this rate-determining conversion at any one time. To us, the reaction cycle would appear less efficient if these states did not have the ability to stabilise protein intermediates at the opposite ring. A similar reasoning may be given to explain the slightly lower affinity of the ADP-bound resting complex which is most likely the predominant conformation in the cell under stress conditions, for example through heat shock [12,32,33]. Under these conditions, the GroEL cycle is slowed because of the accumulation of ADP [42,43], although temperature-induced conformational changes in GroEL may also play a role [44]. It seems attractive to speculate that the resting complex may provide a means for GroEL to stabilise large amounts of unfolded proteins in the cell until the ATP/ADP ratio is restored, no further unfolding occurs and tight binding during hydrolysis is necessary to restore the productivity of the refolding cycle by minimising non-productive losses of bound polypeptide into the bulk solution.

Our study using wild-type GroEL and GroES and physiological concentrations of ATP demonstrated a high affinity of the chaperone machinery for peptides under protein folding conditions. This result raises the possibility that successive structural readjustments during ATP hydrolysis create high affinity states of GroEL for substrates, and therefore complements results of experiments that describe the affinity of GroEL complexes under static conditions [5]. It will be a challenge for further investigations to corroborate this hypothesis and demonstrate the existence of successive state and affinity changes in GroEL during hydrolysis.

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