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Cooperativity in ATP hydrolysis by GroEL is increased by GroES

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The kinetics of ATP hydrolysis by the 'molecular chaperone' GroEL and the inhibition of this hydrolysis by GroES have been studied in more detail. It is shown that the hydrolysis of ATP by GroEL is cooperative with respect to ATP with a Hill coefficient of 1.86 (±0.13). In the presence of GroES, there is an increase in the degree of cooperativity with a Hill coefficient of 3.01 (±0.18). The observed cooperativity is not due to dissociation of the GroEL oligomer into smaller units but more probably involves structural changes within the GroEL oligomer.

GroEL; GroES; Chaperone; ATP hydrolysis; Protein folding; Cooperativity

1. INTRODUCTION

The GroEL and GroES proteins from *E. coli* belong to the family of proteins termed 'molecular chaperones' [1] or polypeptide chain binding proteins [2]. Both GroE proteins are oligomers, GroEL is composed of 14 identical subunits of M_r 57 259 and GroES is composed of 7 identical subunits of 10 497. GroEL and GroES form a complex in the presence of ATP [3,4] and have been demonstrated to promote the folding and assembly of some denatured proteins [5–9]. GroEL exhibits a low ATPase activity [10], requiring potassium ions [11], and this activity is inhibited in the presence of GroES [3,11]. GroES appears to modulate the function of GroEL and it has been proposed that GroES couples K^+-dependent hydrolysis of ATP to the release of the folded target protein from GroEL [11]. This may be achieved by GroES coordinating Mg-ATP-dependent conformational changes in GroEL [9]. In the present study, we report the cooperative nature of ATP hydrolysis by GroEL and the effect of GroES on this hydrolysis. The results are discussed in relation to the MWC model for cooperativity [12].

2. MATERIALS AND METHODS

2.1. *Reagents*

[8-^{14}C]ATP was purchased from Amersham International plc and polyethyleneimine sheets were from Merck.

2.2. *Protein expression and purification*

GroEL and GroES chaperones were expressed in the *E. coli* strain CG1849 (kindly donated by Dr C. Georgopoulos) that contains the GroEL/ES-bearing plasmid pOF39 [13]. Purification of both proteins was achieved using the following procedures. A crude lysate was prepared from *E. coli* CG1849. GroEL and GroES were then precipitated by the addition of ammonium sulphate to 40% [3]. After centrifugation at 18 000 rpm for 30 min, the pellet was resuspended in and dialysed against 50 mM Tris-HCl pH 7.78, 5% v/v glycerol, 2 mM β-mercaptoethanol, 1 mM EDTA and 0.1 M KCl (buffer A). The sample was concentrated and enriched for GroEL by passing it through an Amicon membrane (type XM300, M_r 300 000 cut-off). The flow-through solution was subsequently concentrated and enriched for GroES by passing it through an Amicon membrane (type PM30, M_r 30 000 cut-off). Aliquots of 200 μl were then applied to 38 ml linear glycerol gradients in buffer A. The samples were sedimented at 4°C in a Beckman SW28 swinging bucket rotor at 28 000 rpm for 24 h. GroEL was found predominantly in the middle third of the gradient. These fractions were pooled and dialysed against 20 mM histidine-HCl pH 5.3, 10 mM glucose and 2 mM β-mercaptoethanol (buffer B) and applied to an FPLC MonoQ (Pharmacia) column (Dr A. Girshovich, personal communication). A linear 0–0.5 M NaCl gradient was used to elute GroEL at approximately 0.45 M NaCl. (GroEL purified by this method elutes at the expected salt concentration when applied to a MonoQ column at pH 7.8, as described in ref. [8]). Homogeneous GroEL oligomer, as determined by SDS/PAGE and gel filtration on Superose 6 (Pharmacia) in 50 mM Tris-HCl pH 7.78, 0.5 M KCl and 10 mM glucose (buffer C), was frozen at −70°C with 10% glucose. Concentrations were determined using an extinction coefficient of 23 800 $M^{-1} \cdot cm^{-1}$ for GroEL protomers [11]. (Throughout, the term protomer is used to mean GroEL monomer, since the smallest functional unit with regard to ATP hydrolysis remains to be defined.) GroES was found barely to enter the glycerol gradient. The appropriate fractions were pooled and applied to an FPLC Mono Q (Pharmacia) column in buffer A (minus KCl). A linear 0–0.4 M NaCl gradient was used to elute GroES at approximately 0.2 M NaCl [8]. This was dialysed against buffer B, applied to Mono Q and eluted with a 0–0.35 M linear NaCl gradient. (Dr A. Girshovich, personal communication.) GroES eluted at around 0.15 M NaCl. Pooled fractions were finally applied to a Superose 6 (Pharmacia) column in buffer C. Homogeneous GroES, as determined by gel filtration and SDS/PAGE, was frozen at −70°C with 10% glucose. GroES concentrations were determined using an extinction coefficient of 3440 $M^{-1} \cdot cm^{-1}$ for protomers [11]. GroES protomer is used throughout to mean GroES monomer.

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2.3. *ATPase assays*

GroEL or a GroEL and GroES mixture was incubated in 50 mM Tris-HCl pH 7.78, 10 mM KCl, 10 mM $MgCl_2$ and 10 mM glucose at 25°C. The reaction was initiated by the addition of [8-^{14}C]ATP preincubated at 25°C. The reaction was quenched after various periods of time by adding 4 μl aliquots of the reaction mixture to 5 μl of 0.7 M LiCl, 1 M HCOOH on ice. Samples (2 μl) of the quenched reaction mixture were spotted on polyethyleneimine sheets. Chromatography was performed in 0.7 M LiCl, 1 M HCOOH to separate reaction products. After drying ATP and ADP spots were located, cut out and counted [14,15]. The radioactivity was expressed as the fraction of ATP hydrolysed. The background of 40 cpm was subtracted from all values. Initial velocities for GroEL-catalysed ATP hydrolysis were determined from plots of [ADP]/([ATP]+[ADP]) versus time using data covering the first 5–10% of the reaction.

2.4. *Data fitting*

In Fig. 1 initial velocities are directly fitted, using Kaleidagraph (version 2.1 Synergy Software (PCS Inc.)), to equation (1):

$$V_o=(V_{max}K_i+V_{min}[I])/(K_i+[I]) \quad (1)$$

where V_o is the initial ATP hydrolysis rate, V_{max} and V_{min} are the initial ATP hydrolysis rates in the absence of inhibitor (GroES) and in the presence of an infinite concentration of inhibitor, K_i is the dissociation constant and [I] the concentration of inhibitor. Relative rates for hydrolysis are used here, as we found some variation in the control rate, in the absence of GroES, between experiments.

Initial velocities for ATP hydrolysis by GroEL at various ATP concentrations were fitted directly, as above, to the Hill equation eqn. (2):

$$V_o=K[S]^nV_{max}/(1+K[S]^n) \quad (2)$$

where V_o and V_{max} are the initial and maximal initial ATPase velocities; [S] is the concentration of substrate, in this case ATP; K is the apparent dissociation constant and n the Hill coefficient. Linear plots were obtained by replotting the data according to the logarithmic form of the Hill equation, eqn. (3):

$$\log(V_o/V_{max}-V_o))=\log K+n\log[S] \quad (3)$$

using values for V_{max} obtained from direct fitting to the Hill equation.

Initital rates for ATP hydrolysis by GroEL were also directly fitted, as above, to the MWC equation [12], eqn. (4):

$$V_o=V_{max}(S/K_R)(1+S/K_R)^{N-1}/(L+(1+S/K_R)^N \quad (4)$$

where V_o and V_{max} are the initial and maximal ATPase velocities, S is the concentration of substrate (ATP), K_R is the dissociation constant for ATP from the R form of GroEL, L is the allosteric constant and N the number of ATP binding sites. This version of the MWC model assumes that ATP binds exclusively to the R state.

3. RESULTS

GroEL is shown to exhibit ATPase activity, with k_{cat}=4.4 s^{-1}. This value is at least 40-fold higher than previously reported by Viitanen et al. [11] and 5-fold higher than described by Hendrix, [10] for different conditions. Unfortunately, there is no accurate method yet available for determining the number of active sites in a particular GroEL sample, so errors in the active enzyme concentration may be responsible for the discrepancies in k_{cat} values. It is unlikely that they result from an impurity since the ATPase activity is specifically inhibited by up to 61%, with a change in the observed cooperativity, upon addition of increasing amounts of GroES (Fig. 1). The data, fitted to eqn. (1), give a value for K_i of 0.28 μM.

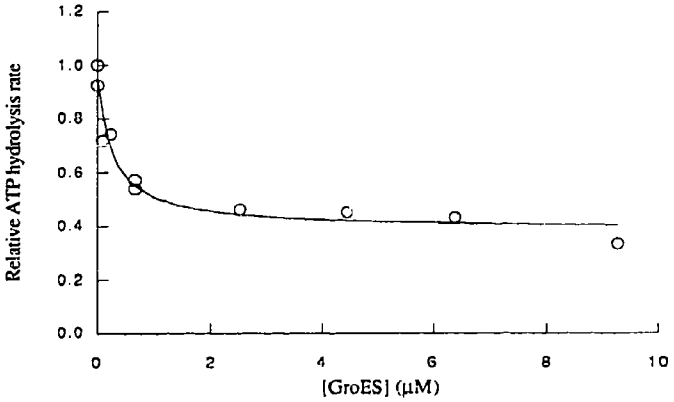

Fig. 1. Inhibition by GroES of GroEL-catalysed ATP hydrolysis. Final ATP and GroEL (protomer) concentrations in this reaction were 320 μM and 0.39 μM respectively, in a total volume of 30 μl. The concentration of GroES was varied from 0–9.65 μM. The ATPase assays were performed as described in section 2.

GroEL ATPase activity was measured at various GroEL concentrations (Fig. 2). The relationship between ATPase activity and GroEL concentration was found to be linear between 0.13 and 0.78 μM GroEL.

A sigmoidal relationship between the initial ATPase velocity and ATP concentration was observed at GroEL concentrations of 0.39 μM and 0.26 μM (Figs 3 and 4). Fitting the data directly to the Hill equation (Eqn. (2)), gives Hill coefficients of 1.86 (±0.13) and 1.80 (±0.13) respectively, indicating that ATP hydrolysis by GroEL is a cooperative process.

Initial velocities for ATP hydrolysis in the presence of 9.65 μM GroES were determined at 2 concentrations of GroEL, 0.39 μM and 0.52 μM. The resulting curves are again clearly sigmoidal and fitting them to the Hill equation (Eqn. 2) yields Hill coefficients of 3.01 (±0.18)

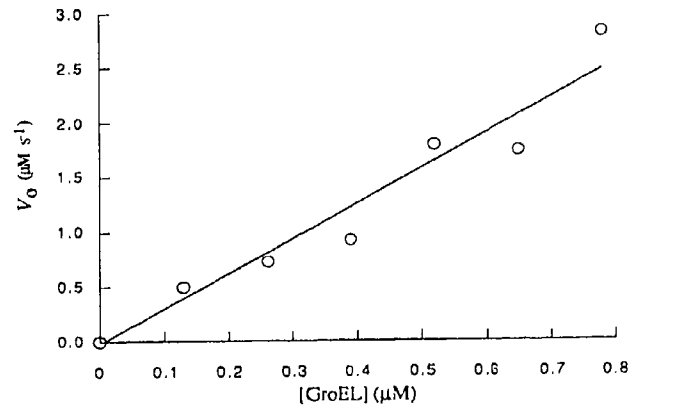

Fig. 2. ATPase activity measured at various GroEL concentrations. The ATP concentration was saturating at 192 μM. The ATPase assays were performed as described in section 2.

and 2.98 (±0.43) as shown in Figs 5 and 6, respectively. Cooperativity with respect to ATP hydrolysis is thus increased in the presence of GroES as reflected by a larger Hill coefficient. The value of V_{max} at saturating GroES concentration (see Fig. 5) decreases to about 40% of that in the absence of GroES (see Fig. 3). This is in good agreement with the degree of inhibition observed by Chandrasekhar et al. [3], but a greater degree of inhibition (≥95%) was observed by Viitanen et al. [11].

The data shown in Figs 3 and 5 were fitted to the MWC equation (Eqn. **(4)**) with N=7 of N=14. These values of N were chosen primarily for reasons of symmetry since GroEL is an oligomer composed of 14 identical subunits. The value of L, the allosteric constant, is increased 15-fold when N=7 and 12-fold when N=14 (Table I). There is relatively little change in the binding constant K_R in the presence or absence of GroES (Table I).

Table I

Analysis of Cooperativity by the MWC Model[a]

MWC parameters	0.39 μM GroEL	0.39 μM GroEL 9.65 μM GroES
N=7		
V_{max} (μM s^{-1})	2.09 ± 0.10	0.69 ± 0.03
K_R (μM)	47.2 ± 5.4	57.5 ± 8.4
L	10.1 ± 4.2	149 ± 95
N=14		
V_{max} (μM s^{-1})	2.30 ± 0.12	0.8 ± 0.06
K_R (μM)	64.8 ± 7.1	105 ± 18.3
L	26.9 ± 15.1	325 ± 330

[a]Estimates for V_{max}, K_R and L obtained after fitting the data shown in Figs. 3 and 5 to the MWC model (eqn. **(4)**).

4. DISCUSSION

Electron microscopy reveals that the GroEL oligomer is composed of 2 rings of heptamers stacked one on top of the other [10,16]. GroES also appears as a heptameric ring that can interact with one GroEL oligomer in the presence of ATP [3]. This interaction partially inhibits ATP hydrolysis by GroEL (Fig. 1). It is unlikely that the partial inhibition observed is due to the presence of a mixed population of GroEL, with some fraction sensitive to GroES inhibition and the remainder insensitive, as there are very different kinetics in the presence and absence of saturating GroES.

The data presented in Figs 3–6 for the Hill equation (Eqn. **(2)**). This equation gives a quantitative measure of cooperativity but is essentially model-independent. There are several models to explain cooperativity, the MWC model [12], the KNF model [17] and a general scheme [18] of which the MCW and KNF models are limiting cases. They all require that the protein has a transition between 2 (or more) states, but they differ in the pathway between states. An equilibrium between the GroEL oligomer and smaller units would be extremely sensitive to changes in GroEL concentration and result in a non-linear relationship between GroEL concentration and ATPase activity. The linear relationship shown in Fig. 2 indicates that dissociation of the GroEL oligomer is probably not associated with the transition between different states that gives rise to cooperativity.

For simplicity we have chosen the MWC model [12] to analyze the cooperativity observed in ATP hydrolysis by GroEL. According to this model, there is an equilibrium between an inactive (tense (**T**)) state and an active (relaxed (**R**)) state of GroEL. Binding of ATP to the **R** state is tighter than to the **T** state, and so in the presence of ATP the equilibrium shifts in favour of the **R** state. In accordance with this model, there must be more than one binding site for ATP on GroEL to account for the cooperativity. Our data indicate that there are at least

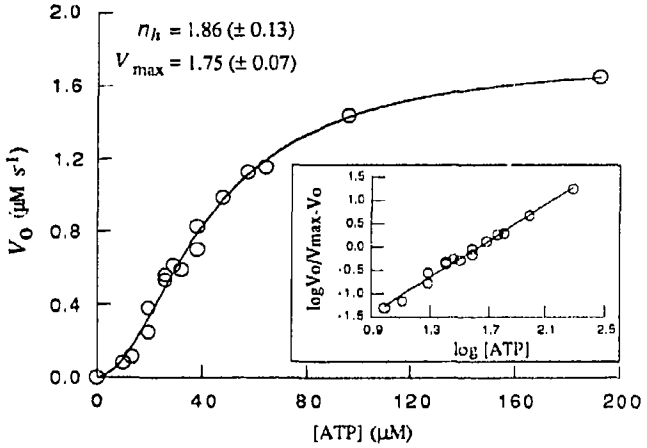

Fig. 3. ATPase activity of GroEL measured at different ATP concentrations. The GroEL concentration was 0.39 μM. The inset shows a Hill plot of the data shown in the Figure. The ATPase assays were performed as described in section 2.

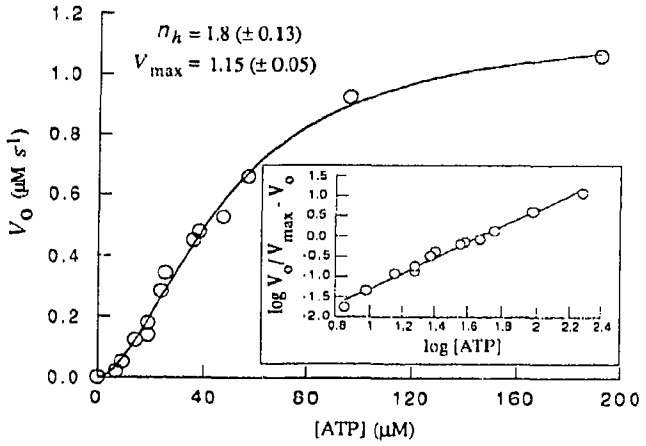

Fig. 4. ATPase activity of GroEL measured at different ATP concentrations. The GroEL concentration was 0.26 μM. The inset shows a Hill plot of the data shown in the Figure. The ATPase assays were performed as described in section 2.

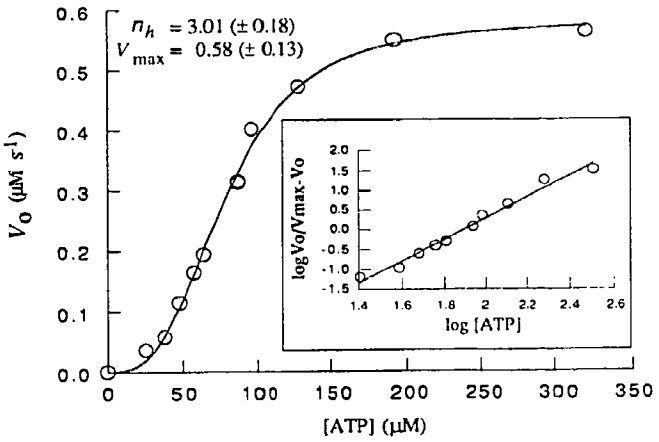

Fig. 5. ATPase activity of GroEL measured in the presence of GroES at different ATP concentrations. The GroEL and GroES concentrations were 0.39 μM and 9.65 μM respectively. The inset shows a Hill plot of the data shown in the Figure. The ATPase assays were performed as described in section 2.

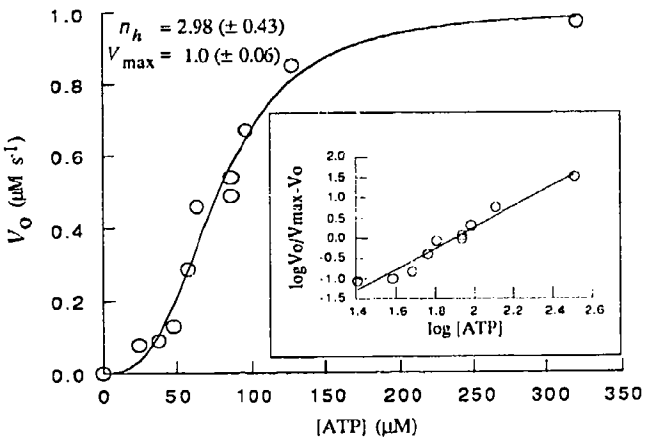

Fig. 6. ATPase activity of GroEL measured in the presence of GroES at different ATP concentrations. The GroEL and GroES concentrations are 0.52 μM and 9.65 μM respectively. The inset shows a Hill plot of the data shown in the Figure. The ATPase assays were performed as described in section 2.

3 ATP binding sites on GroEL, since in the presence of GroES the Hill coefficient is found to be about 3. As yet, the stoichiometry of ATP binding to GroEL has not been determined but for reasons of symmetry an assumption of N equal to either 7 or 14 is reasonable. Accordingly, the data presented in Fig. 3 were fitted to the MWC equation (eqn. (4)) with N equal to 7 or 14 and a good fit was obtained in both cases (these fits are not shown). The values obtained for L (the allosteric constant) from these fits are 10 and 27 respectively, depending on the value for N (see Table I). This may reflect significant populations of both the **T** and **R** states under these conditions, indicating that they have similar free energies. The values for L shown in Table I are very low compared to those found for other proteins with fewer subunits, for example haemoglobin, where L is equal to 3×10^5 [19]. The presence of many subunits in GroEL may amplify the effect of cooperativity such that sifnificant values for the Hill coefficient, n, are obtained with low L values.

GroES inhibits ATP hydrolysis by GroEL (Fig. 1). It is not clear whether ATP hydrolysis is required for the formation of the GroEL/ES complex. The complex does not form in the presence of β,γ-methyleneadenosine 5'-triphosphate [3], but it is reported that ADP does stabilize the GroEL/ES complex [7]. These observations appear consistent with the suggestion that continued ATP hydrolysis is not required to maintain the GroEL/ES complex [11] but one round of hydrolysis may be involved in complex formation. In the experiments described here, we assume that the GroEL/ES complex forms rapidly and remains largely stable, so that dissociation and reformation of the complex, with any accompanying ATP hydrolysis, would be kinetically negligible during the course of the experiments.

Experiments in which ATP hydrolysis by GroEL was measured in the presence of GroES show that there is an increase in cooperativity, as reflected by a larger Hill constant (Figs 5 and 6), suggesting that GroES is an allosteric inhibitor. At saturating levels of GroES (Figs 5 and 6) singificant ATPase activity remains and the Hill number does not rise above 3. If GroES were binding exclusively to the **T** state then, with increasing levels of GroES, the ATPase activity should reduce to zero and the Hill coefficient should tend towards the number of ATP binding sites, which we surmise may be of 7 or even 14. It therefore seems more reasonable to propose that GroES can bind to the **T** and **R** states of GroEL, although somewhat better to the **T** state. At saturating GroES concentrations, the **T/R** ratio L is, therefore, slightly increased. The reduction in k_{cat} observed in the presence of GroES may be due to some alteration in the conformation of the ATPase active sites or to GroES sterically blocking some ATP binding sites or to a combination of both effects. The data in Fig. 5 for ATP hydrolysis in the presence of GroES were also fitted to the MWC equation (Eqn. (4)) and good fits were obtained for N equal to 7 and 14 (these fits are not shown). The fact that the GroEL/ES complex is still able to hydrolyse ATP (see Fig. 1), albeit more slowly than GroEL alone, is consistent with there hardly being any change in the K_R value of ATP (Table I) in the presence of GroES and with the explanation that some of the ATP binding sites on GroEL are blocked by the GroES 7-mer. The relatively small increase in the value of L, the allosteric constant (Table I), obtained in the presence of GroES may be due to a very small difference between the binding affinity of GroES for the **T** and **R** states of GroEL.

Results described here show that ATP hydrolysis by GroEL is a cooperative process and that in the complex with GroES the degree of cooperativity is increased. It has been suggested that the role of GroES is to couple

the K^+-dependent hydrolysis of ATP to the release of 'substrate' protein from GroEL [11]. Our results now suggest that this coupling involves increased cooperativity with respect to ATP hydrolysis by GroEL, accompanied by a decrease in the V_{max} for this hydrolysis. More recently it has been proposed that a quaternary structural change in GroEL is driven by ATP hydrolysis [20] and that GroES coordinates Mg-ATP-dependent conformational changes of GroEL [9]. The quantitative data for ATP hydrolysis presented in this paper support these proposals [9,20].

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