

The role of ATP hydrolysis in the function of the chaperonin GroEL: dynamic complex formation with GroES

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Abstract In order to understand the role of ATP hydrolysis of the chaperonin GroEL during protein folding, we have studied GroEL–GroES complex formation in the presence of ATP or ADP by using capillary electrophoresis and surface plasmon resonance. Capillary electrophoresis analysis showed that the GroEL 14-mer and GroES 7-mer formed a 1:1 complex in the presence of ATP. In the presence of ADP, both the association and dissociation rates of the complex were slower by about one order of magnitude than the rates in the presence of ATP at 25°C. The implications of such a stable complex on the overall mechanism of chaperonin function are discussed.

Key words: Chaperonin; GroEL–GroES complex formation; ATP hydrolysis; Capillary electrophoresis; Surface plasmon resonance

1. Introduction

The chaperonin GroE (GroEL (14-mer) and GroES (7-mer)) from *Escherichia coli* has been shown to facilitate protein folding in vivo and in vitro [1]. The functional mechanism of GroE can be divided broadly into two parts; binding of protein folding intermediates prone to aggregation, and the controlled release of these intermediates to allow maximum refolding yields. In the former step, GroEL recognizes a folding intermediate and binds it specifically by hydrophobic interactions [2,3]. By forming this complex, the folding intermediate is stabilized and irreversible aggregation is suppressed. In the latter step, which usually occurs when ATP is added, an efficient release of the intermediate occurs and the native protein is formed. In this step, GroES protein plays an important role in assisting the function of GroEL [4,5].

The role of ATP hydrolysis during the so-called 'chaperonin cycle' remains a mystery. In many protein refolding reactions, the addition of various non-hydrolyzable nucleotides such as ATP- γ -S [6–8], AMP-PNP [7–9], or ADP [4,7,10–12], is sufficient to release the bound intermediates, as well as ATP. This fact strongly suggests that the binding of nucleotides to GroEL is important and that the energy derived from ATP hydrolysis may not be directly involved in the mechanism. In order to understand the role of ATP hydrolysis during chaperonin function, we have studied in detail the association and dissociation of GroEL and GroES in the presence of ATP and ADP. Free-

solution capillary electrophoresis analysis showed that GroEL (14-mer) and GroES (7-mer) formed a 1:1 complex in the presence of 2 mM ATP. The kinetics of the specific interactions between GroEL and GroES in the presence of 2 mM ATP or ADP were also studied by using a BIAcore system (Pharmacia). The rate constants for the association and dissociation of the GroEL–GroES complex in the presence of ADP were both slower by about one order of magnitude compared to the rates in the presence of ATP. These results are discussed in light of previous experiments regarding the nucleotide specificity of chaperonin function.

2. Materials and methods

2.1. Proteins

GroEL and GroES proteins were initially purified from a GroE-overproducing strain, *E. coli* DH1/pKY206 according to the method of Kubo et al. [4] and further purified by performing gel-filtration chromatography on an FPLC column (Pharmacia Superdex 200 HR) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing 100 mM KCl, 4 mM MgCl₂, 1 mM β -mercaptoethanol, and 0.6 mM ATP. The GroEL–GroES complex was formed prior to application to this column in the presence of 4 mM ATP, as reported previously [13]. GroEL and GroES proteins were separated by a second gel-filtration in the absence of ATP. This additional purification step was very effective, especially for GroES protein. The concentration of GroEL was determined spectrophotometrically on a Hitachi U-2000 spectrometer, using an absorption coefficient of $A_{1\text{cm}}^{1\%} = 2.36$ at 277 nm [7]. The concentration of GroES was determined by the method of Bradford [14], using bovine serum albumin as a standard.

2.2. Capillary electrophoresis analysis

Free-solution capillary electrophoresis analysis was performed on a Quanta 4000E system (Waters) with a fused silica capillary tube ($\varnothing 75 \mu\text{m} \times 45 \text{ cm}$) at 25°C. The buffer used was 50 mM MOPS-KOH, pH 7.0, containing 10 mM Mg(CH₃COO)₂, 10 mM KCl, 0.5 M trimethylammoniumpropanesulfonate (which suppresses non-specific adsorption of protein to the capillary surface), and either 2 mM ATP or ADP. The sample was applied by hydrostatic method and typically the amount of protein applied was less than 50 ng per run. A constant voltage of 15–17 kV (inlet reservoir; positive, outlet reservoir; negative, current; 90–100 μA) was applied and the changes in absorbance at 185 nm were monitored as a function of time.

2.3. Association and dissociation kinetic analyses

Association and dissociation analyses of GroEL and GroES in the presence of ATP or ADP were performed on a BIAcore system (Pharmacia) at 25°C. Purified GroES protein (1 mg/ml) in 10 mM acetate buffer at pH 4.7 was covalently immobilized at 25°C for 7 min onto the dextran matrix of the sensorchip (CM5) surface, which had been activated with a 1:4 (mol/mol) mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pharmacia). The excess active groups on the dextran matrix were blocked with 1 M ethanolamine-HCl (pH 8.8). The area concentration of immobilized GroES protein was 3.6 ng/mm² (the increase in resonance units (ΔRU) was 3600). The buffer used for the kinetic analyses

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Abbreviations: MOPS; 3-morpholinopropanesulfonic acid.

was 50 mM MOPS-KOH at pH 7.0 containing 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$ and 100 mM KCl. Various concentrations of GroEL protein were added to this buffer containing either 2 mM ATP or ADP and injected over the sensor chip surface at a flow rate of 5 $\mu\text{l}/\text{min}$. After each run, the system was regenerated by alternately washing with buffer plus ATP and buffer minus ATP. Complete regeneration was achieved after two cycles of this washing protocol (5 min/cycle).

3. Results and discussion

3.1. Complex formation of GroEL and GroES in the presence of ATP or ADP

It has been shown that GroEL and GroES proteins form a complex in the presence of either ATP [15] or ADP [16–18]. Recently, it was also reported that a 1:2 complex (football shape) of GroEL and GroES was formed in the presence of ATP [19,20] and this conformation was incorporated into an elegant mechanism describing interactions between GroEL, GroES, and ATP [21]. The electron microscopic data, however,

essentially provides a static snapshot of the chaperonin conformations and does not yield data regarding the specific steps leading to these conformations. Therefore, in order to confirm the dynamics of complex formation, we have used free-solution capillary electrophoresis analysis. Capillary electrophoresis separates various proteins and their conformers by their net charge and/or molecular size in a process called electroosmosis.

As shown in Fig. 1a, in the absence of nucleotide, GroEL and GroES proteins migrated separately. GroES protein migrated faster than GroEL, indicating that the net negative charge of GroES is less than that of GroEL at pH 7.0. This is consistent with the elution order from the anion-exchange chromatography column in the purification procedure. However, as shown in Fig. 1b, when GroES was mixed with excess amounts of GroEL in the presence of 2 mM ATP, the GroES peak could not be detected, indicating that GroES binds to GroEL. Under these conditions GroEL and the GroEL–GroES complex were indistinguishable. Interestingly, the peak corresponding to GroEL/GroEL–GroES was markedly sharper than the GroEL peak seen in the absence of nucleotides. When the amount of GroES exceeded the amount of GroEL, the GroES peak reappeared. Titration of GroEL (14-mer) with GroES (7-mer) revealed a 1:1 relationship under our experimental conditions. Although at first glance this result seems contradictory with the electron microscopic results [19,20], the football-shape complex of GroEL and GroES is thought to be present only transiently during the chaperonin ATP-hydrolysis cycle [15,21]. The relationship between the rates of interaction and the time scale of the experiment [22] may therefore be limiting our observations. This 1:1 complex of GroEL and GroES was also observed in the presence of 2 mM ADP (data not shown).

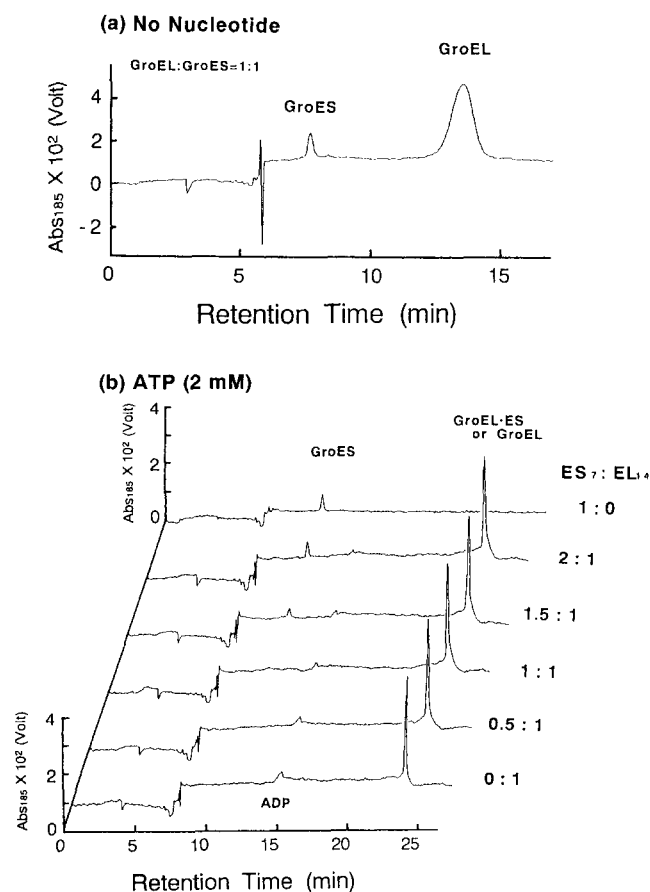


Fig. 1. Free-solution capillary electrophoresis analysis of GroEL and GroES proteins in the absence (a) and presence (b) of ATP. Various molar ratios of purified GroEL and GroES were mixed in the buffer with (b) or without (a) 2 mM ATP and allowed to stand at 25°C for 2–10 min prior to application of the sample. About 30–50 ng protein was applied per analysis. The absorbance at 185 nm was monitored as changes in photomultiplier voltage. The negative peak at 4 min was due to the buffer in which the proteins were dissolved, and the small peak at 14 min (in (b)) was from ADP that had been hydrolyzed by GroEL in the sample solution before application. The sharp spiked peak observed at 6 min in (a) or 8 min in (b) is due to neutral net-charge solutes, and substances of positive and negative net charge are eluted before and after these peaks, respectively.

3.2. Kinetics of association and dissociation of the GroEL–GroES complex in the presence of ATP and ADP

In order to determine how quickly the GroEL–GroES complex is formed and how stable the complex is in the presence of ATP or ADP, we have studied the kinetics of GroEL–GroES complex formation by using the BIAcore instrument, which provides a real-time analysis of specific protein–protein interactions [23–25]. Initially, we attempted to immobilize GroEL onto the sensorchip but failed due to aggregation which was observed in the acidic pH region during the immobilization step. GroES protein, however, was successfully immobilized at pH 4.7. Although denaturation of GroES could be a problem in subsequent experiments, separate experiments showed that unfolded GroES could refold spontaneously and restore its function (unpublished results), arguing favorably for a regeneration of active GroES after immobilization.

Fig. 2a shows the association and dissociation profiles of GroEL to GroES which had been immobilized on the sensorchip. In the presence of ATP, GroEL associated and dissociated from GroES very quickly. In sharp contrast, in the presence of ADP, GroEL associated and dissociated very slowly. Specifically, in the presence of ADP the dissociation rate of the complex to GroEL and GroES was about 30 times slower than in the presence of ATP. By varying the concentration of GroEL (Fig. 2b), the association rate constants in the presence of 2 mM ATP or ADP were obtained and are shown in Table 1 with the dissociation rate constants. In the case of ATP, the hydrolysis of the nucleotide may pose a problem in determining accurately the dissociation rate constant, since the apparent rate constant

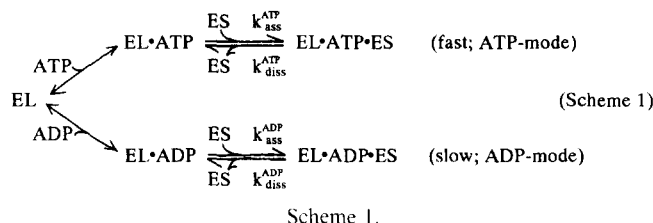
would include the exchange of the hydrolyzed product, ADP, with ATP. To evaluate this possibility, we formed chaperonin complexes in the presence of ADP, and dissociated them in the presence of ATP. The rationale of this experiment was to observe any differences in the dissociation rate constant which should occur if the contribution of nucleotide exchange was maximized. However, as shown in Fig. 2a, the rate constants of the two experiments were indistinguishable ($2.6 \times 10^{-3} \text{ s}^{-1}$). This indicates that the contribution of nucleotide exchange in the dissociation rate constant is negligible.

The association rate of GroEL and GroES in the presence

of ATP was about $10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value is consistent with the association rate constant of GroEL and GroES proteins ($1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) determined by Jackson et al. [18] and also seems to be quite reasonable when compared with other specific protein-protein interactions [26] (e.g. $3.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the subunit association of triosephosphate isomerase [27]). The value of the dissociation equilibrium constant (K_{diss}) in the presence of ADP was also consistent with values (~ 3 –26 nM) from other studies [17,18].

3.3. Role of ATP hydrolysis in the chaperonin function

As reported by Todd et al. [21,28] and Gray and Fersht [29], GroEL hydrolyses ATP and its hydrolytic activity is regulated by GroES. In the present study, we could observe interactions between GroEL and GroES in the presence of ATP and ADP, suggested to be important in the chaperonin functional cycle [4,15]. From the results obtained in the present experiments, the interactions of GroEL and GroES proteins can be expressed as shown in Scheme 1.



where $\text{EL} \cdot \text{ATP} \cdot \text{ES}$ and $\text{EL} \cdot \text{ADP} \cdot \text{ES}$ are the complexes of GroEL and GroES proteins in the presence of ATP and ADP, respectively, and $k_{\text{ass}}^{\text{ATP}}$, $k_{\text{diss}}^{\text{ATP}}$ and $k_{\text{ass}}^{\text{ADP}}$, $k_{\text{diss}}^{\text{ADP}}$ are the association and dissociation rate constants in the presence of ATP and ADP, respectively.

In the presence of ATP, GroEL binds GroES to form $\text{EL} \cdot \text{ATP} \cdot \text{ES}$ quickly but also dissociates it quickly (ATP-mode

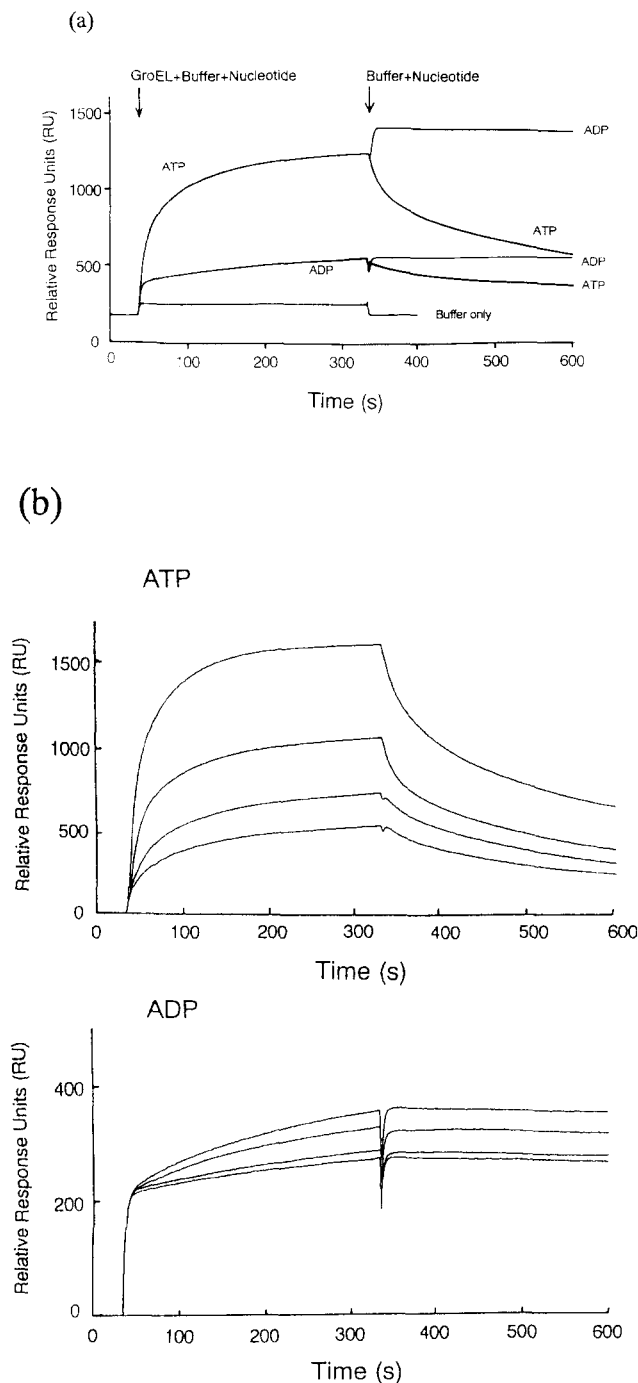


Fig. 2. Sensorgrams of the binding and release of GroEL with immobilized GroES in the presence of 2 mM ATP and ADP at 25°C. (a) GroEL at constant concentration (133 nM): The running buffer used for the experiments was 50 mM MOPS-KOH at pH 7.0, containing 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$ and 100 mM KCl. The association phase was started by injecting running buffer containing 133 nM GroEL and 2 mM nucleotide at the time indicated by the first arrow in the figure. At the time indicated by the second arrow, the dissociation reactions were started by switching the flow solution to running buffer containing 2 mM nucleotide. The nucleotides used were indicated in the figure. (b) GroEL at variable concentrations: In order to obtain the association rate constants GroEL was injected at various concentrations in the presence of ATP (upper panel, from bottom trace: 38, 57, 133, 247 nM) and at various concentrations in the presence of ADP (lower panel, from bottom trace: 38, 57, 95, 133 nM). The total response changes of the association and dissociation for all experiments were described by a single-exponential decay term, and the association rate constants were obtained by plotting

$$\left\{ \partial \left(\frac{d\text{RU}}{dt} \right) / \partial (\text{RU}) \right\}$$

against the concentrations of GroEL (Table 1). The data from $t = 50$ to 250 s and $t = 350$ to 550 s were used for analyzing the association and dissociation reactions, respectively. When only running buffer containing either ATP or ADP was applied, the increases in response units were about 100 and 200 RU, respectively.

Table 1

Kinetic parameters of GroEL and GroES interactions in the presence of ATP and ADP at 25°C

Nucleotide (2 mM)	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_{diss} (nM)
ATP	1.7×10^5	2.8×10^{-3}	16.4
ADP	1.3×10^4	1.1×10^{-4}	8.3

in Scheme 1). The apparent ATP-hydrolysis rate (k_h) of EL·ATP·ES under steady-state conditions was reported to be about 0.02 [18]–0.04 [17] s^{-1} at 25°C. Since the rate of ATP hydrolysis is greater than the dissociation rate of the EL·ATP·ES complex ($k_h > k_{\text{diss}}^{\text{ATP}} = \sim 0.003 \text{ s}^{-1}$, Table 1), the ATP bound to GroEL will be hydrolyzed to ADP before the dissociation of GroES. In the presence of ATP, the EL·ADP·ES complex was seen to dissociate rapidly (Fig. 2a), allowing a regeneration of the EL·ATP·ES form. This dynamic ‘ATP-mode’ reaction in which the binding and release between GroEL and GroES proteins are repeating quickly is most likely the main mechanism responsible for chaperonin function.

In the presence of ADP, GroEL associates to GroES slowly but hardly dissociates from GroES once the GroEL–GroES complex is formed (ADP-mode in Scheme 1), indicating that EL·ADP·ES is very stable in the absence of ATP. Therefore, once the ATP of the EL·ATP·ES form is hydrolyzed, the resulting EL·ADP·ES form would not undergo fast dissociation if ATP is not present. It is known that during heat shock the concentration of ATP in vivo decreases rapidly and the ratio of ADP to ATP increases [30]. Under such conditions, the EL·ADP·ES form may be the predominant conformation in the cell.

In our studies regarding the refolding of tryptophanase [7], enolase [4], and other enzymes [10] in the presence of GroEL and GroES, we reported that the addition of ADP was sufficient to initiate release of protein folding intermediates. This result has since been confirmed in a number of other laboratories using various target proteins [11,12]. This fact strongly suggests that the EL·ADP·ES form is very important for chaperonin function. The results obtained in the present study indicate that in the presence of ADP only, GroEL and GroES undergo slow binding and release interactions. Further experiments will be directed at probing the relationship between the folding pathways of various target proteins and the rate constants elucidated in the present study.

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