Hydrolysis of Adenosine 5′-Triphosphate by *Escherichia coli* GroEL: Effects of GroES and Potassium Ion

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ABSTRACT: The potassium-ion activation constant (K_{act}) for the ATPase activity of *Escherichia coli* chaperonin groEL is inversely dependent upon the ATP concentration over at least 3 orders of magnitude. The ATPase activity shows positively cooperative kinetics with respect to ATP and K^+. Both the $K_{0.5}$ for ATP and cooperativity (as measured by the Hill coefficient) decrease as the K^+ concentration increases. Equilibrium binding studies under conditions where hydrolysis does not occur indicate that MgATP binds weakly to groEL in the absence of K^+. In the absence of groES, the K^+-dependent hydrolysis of ATP by groEL continues to completion. In the presence of groES, the time course for the hydrolysis of ATP by groEL becomes more complex. Three distinct kinetic phases can be discerned. Initially, both heptameric toroids turn over once at the same rate that they do in the absence of groES. This leads to the formation of an asymmetric binary complex, $groEL_{14}$–$MgADP_7$–$groES_7$, in which 7 mol of ADP is trapped in a form that does not readily exchange with free ADP. In the second phase, the remaining seven sites (containing readily exchangeable ADP) turn over, or have the potential to turn over, at the same rate as they do in the absence of groES, so that the overall rate of hydrolysis is maximally 50%. These remaining sites of the asymmetric binary complex do not hydrolyze all of the available ATP. Instead, the second phase of hydrolysis gives way to a third, completely inhibited state, the onset of which is dependent upon the relative affinities of the remaining sites for MgATP and MgADP. This fully inhibited state may be returned to the 50% inhibited state by increasing the ATP/ADP ratio or by increasing the relative affinity of the available sites for MgATP relative to MgADP (by increasing the K^+ concentration). At low K^+ concentrations (1 mM) it is possible to create the completely inhibited state in the absence of free MgADP; the apparent affinity of this asymmetric binary complex for MgATP is reduced 1 order of magnitude.

The chaperonins are ubiquitous and seemingly indispensable proteins which are believed to facilitate the proper folding of some proteins (Gething & Sambrook, 1992). There are two chaperonin proteins, typified by those from *Escherichia coli*: groEL and groES. *In vitro* the chaperonins enhance the yield of folded protein, most often without increasing the rate of the folding process. In some, but not all, cases the formation of the native protein requires the presence of groES and the hydrolysis of ATP (Jaenicke, 1993; Lorimer et al., 1993). We have previously suggested that groES acts as a "coupling factor" linking the hydrolysis of ATP by groEL to the release of the target protein in a form that is committed to the native state (Viitanen et al., 1990). However, the manner in which this is accomplished is quite unknown.

The subunits of groEL, a 14-mer, and groES, a 7-mer, are organized as heptameric toroids. GroEL is a double toroid (Hohn et al., 1979; Hendrix, 1979), while groES is a single toroid (Chandrasekhar et al., 1986; Weaver et al., 1993). GroEL catalyzes the hydrolysis of ATP in a K^+-dependent manner (Viitanen et al., 1990). In the presence of MgATP (or MgADP) groES and groEL form an asymmetric binary complex, $[groEL–groES]_7$–$[groEL]_7$, inhibiting the ATPase activity of groEL (Chandrasekhar et al., 1986; Viitanen et al., 1990; Gray & Fersht, 1991; Bochkareva et al., 1992). Negatively stained images of this complex have recently been reported (Saibil et al., 1991; Langer et al., 1992). The extent of the inhibition of groEL's ATPase activity by groES varies, with some investigators (Viitanen et al., 1990; Langer et al., 1992) reporting complete inhibition, and others (Chandrasekhar et al., 1986; Gray & Fersht, 1991; Jackson et al., 1993), only a maximum of 50% inhibition. To resolve these differences, we have investigated the influence of K^+ and nucleotide concentrations on the *complete* time course of ATP hydrolysis by groEL, in the presence and absence of groES. The results indicate that ATP hydrolysis by the chaperonin complex occurs in an asymmetric manner in which one of the heptameric toroids of groEL is completely inhibited while the other hydrolyzes ATP with significantly altered kinetic properties.

EXPERIMENTAL PROCEDURES

Purification of GroEL and GroES. The chaperonin proteins were overexpressed in *E. coli* containing the plasmid pGroESL (Goloubinoff et al., 1989) and purified using modifications of published procedures (Hendrix, 1979; Chandrasekhar et al., 1986). Cells were ruptured using a French pressure cell (>16 000 psi). After centrifugation (10^5g, 30 min), the supernatant was applied to a DEAE-Sephadex column (4 L) equilibrated with buffer A (50 mM Tris, pH 7.2, 0.1 mM EDTA, and 1 mM DTT) and developed with a linear gradient of 0–1 M NaCl (in 16 L) to provide fractions enriched for groES and groEL eluting at 0.2 and 0.35 M NaCl, respectively.

GroEL-enriched fractions were applied to a Sephacryl S-300 column (6 L) equilibrated with buffer A. GroEL, eluting in the void volume, was next applied to a Mono-Q HR16/10 column (25 °C) equilibrated with 50 mM MES, pH 6.0, 1 mM EDTA, and 1 mM DTT and was eluted with a gradient 0–1 M in NaCl. In our hands, the groEL eluting from this column contained trace amounts of other ATP-utilizing

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enzymes. Therefore, the groEL (already a single band on SDS–PAGE) was further purified on a hydroxyapatite column (Bio-Rad 25-mL BioGel HPT) that was developed with a linear gradient of 5–400 mM sodium phosphate, pH 7.0, containing 10 μM $CaCl_2$. GroEL elutes at ~130 mM P_i, and the major contaminants are an early shoulder on this peak. The fractions free of contaminants were pooled, concentrated to >1 mM, and stored in 20 mM Tris-HCl, 0.1 mM EDTA, and 1 mM DTT at –80 °C.

GroES from the DEAE-Sepharose column was applied to a Mono-Q column equilibrated with buffer A and eluted with a 0–1 M NaCl gradient. Fractions containing groES fractions (~50% pure) were further purified by Mono-S chromatography in 50 mM sodium succinate and 0.1 mM EDTA , pH 4.6, with a 0–1 M NaCl gradient. GroES purified in this manner appears homogeneous by SDS–PAGE yet still has contaminating ATPase activity. This contaminant was removed as a shoulder eluting immediately following the groES peak on a hydroxyapatite column, using buffers described above.

Unless stated otherwise, groEL and groES concentrations refer to the concentration of protomer and were determined by quantitative amino acid analysis.

Since the uncoupled ATPase activity of groEL is slow compared to many other ATPases (~5 min^{-1}; Viitanen et al., 1990), it was essential to remove trace amounts of other ATP-using enzymes. In addition to contaminating ATPases, another interfering activity resulted in the formation of a high-MW polymer of poly-A. This material (λ_{max} = 260 nm) is easily detected in the void volume during gel filtration chromatography on a TSK-4000 column or by failure to obtain a stoichiometry of 1 mol of $^{32}P_i$ per mol of [γ-^{32}P]ATP during the groEL ATPase assay.

ATPase Assays. ATP hydrolysis was assayed using orthophosphate–molybdate extraction of ^{32}P-labeled phosphate, as described previously (Viitanen et al., 1990). All ATPase assays were performed at 25 °C in standard assay buffer (100 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA), with various concentrations of KCl and nucleotide. Control reactions were run under all conditions in the absence of groEL, and extracted phosphate was subtracted as background.

Other methods are described in the captions to the figures.

DATA ANALYSES

Initial Rates. Initial rates were measured when <5% of the total ATP was hydrolyzed, using linear regression analysis of multiple time points ($n > 4$). Rates determined as a function of KCl or ATP concentration were fit to the Hill equation (Hill, 1925):

$$\frac{v}{V_{max}} = \frac{[S]^n}{K' + [S]^n}$$

where v is the observed rate of ATP hydrolysis, $V_{max} = k_{cat}[groEL_t]$, [S] is the ATP concentration, n is the Hill coefficient, and $K' = [S]_{0.5}^n$, using a BASIC program (Schreiner et al., 1985) which employs the Marquardt algorithm (Marquardt, 1963) to fit by least-squares a set of data points to a curve.

Kinetics of Formation of the Asymmetric Binary Complex $GroEL_{14}$–$MgADP_7$–$GroES_7$. For a simple kinetic decay process the time course for product formation is described as follows:

$$[product]_t - [product]_0 = \Delta[product] = V_f t + (V_i - V_f)(1 - e^{-k_{dec}t})/k_{dec}$$

where V_i and V_f are the initial and final rates of product formation, t is time, and k_{dec} is the rate constant describing the decay of V_i to V_f. Generally, for the binding of an inhibitor, $V_f = V_i \beta$, where β represents the proportion of unoccupied sites. In the case of the binding of groES to groEL, the maximum site occupancy is 0.5 and $V_f = V_i/2$. Hence,

$$(\text{no. of turnovers})_t = \frac{\Delta[product]}{[\text{active sites}]} = \frac{k_{cat}t}{2} + \frac{k_{cat}(1 - e^{-k_{dec}t})}{2\,k_{dec}} \quad (1)$$

where $k_{cat} = V_i/[\text{active sites}]$.

Interactions of GroEL and GroES. For analysis of the interaction of groES with groEL, data were fitted to eq 2,

$$v/V_{max} = \frac{[S]^n(1-\alpha)}{K' + [S]^n} + \frac{[S]^n\alpha}{2(K'' + [S]^n)} \quad (2)$$

$$\alpha = \{K_d + [groES_t] + [groEL_t] \pm [(K_d + [groES_t] + [groEL_t])^2 - 4[groEL_t][groES_t]]^{1/2}\}/2[groEL_t]$$

where α = [groEL–groES]/[$groEL_t$], [$groES_t$] = total concentration of groES in the system, [$groEL_t$] = total concentration of groEL, [groEL–groES] = concentration of the asymmetric binary complex, $K'' = [S]_{0.5}^n$ associated with the asymmetric binary complex, and K_d is the equilibrium dissociation constant between groEL and groES. Equation 2 recognizes that the observed ATPase activity of groEL in the presence of groES is the sum of the activity of the remaining $groEL_{14}$ (corresponding to the first term on the right hand side of the equation) *plus* the activity of the asymmetric binary complex, $groEL_{14}$–$MgADP_7$–$groES_7$ (corresponding to the second term on the right hand side of the equation). Each term on the right-hand side of eq 2 is similar in form to the equation developed by Williams and Morrison (1979) to describe the interaction of a tight-binding inhibitor to an enzyme, with the added complication that only half of the sites interact.

Experimentally, two limiting conditions are recognized. (i) At 100 mM K^+ and 100 μM ATP, the conditions used in Figure 3A, $[S]^n >> K' \approx K''$ (M. Todd, unpublished data; Gray & Fersht, 1991[1]), and eq 2 simplifies to $v/V_{max} = 1 - \alpha/2$. In the limit, when [$groES_t$] >> [$groEL_t$], $\alpha = 1$, and v/V_{max} further reduces to 1/2, the observed phase II condition (see below). (ii) At 1 mM K^+ and 100 μM ATP, the conditions used in Figure 4B, $K'' >> K' \sim [S]^n$, and equation 2 simplifies to

$$\frac{v}{V_{max}} = \frac{[S]^n(1-\alpha)}{K' + [S]^n} + \frac{\alpha[S]^n}{2K''}$$

In the limit, when [$groES_t$] >> [$groEL_t$], $\alpha = 1$, and v/V_{max} further reduces to $[S]^n/2K''$, which approaches 0, the observed phase III condition.

[1] Gray and Fersht (1991) found $K' \sim$ 45–65 μM, and $K'' \sim$ 55–105 μM when assaying groEL ATPase in buffers containing 10 mM KCl, in agreement with our simplifying assumption that $K' \sim K''$.

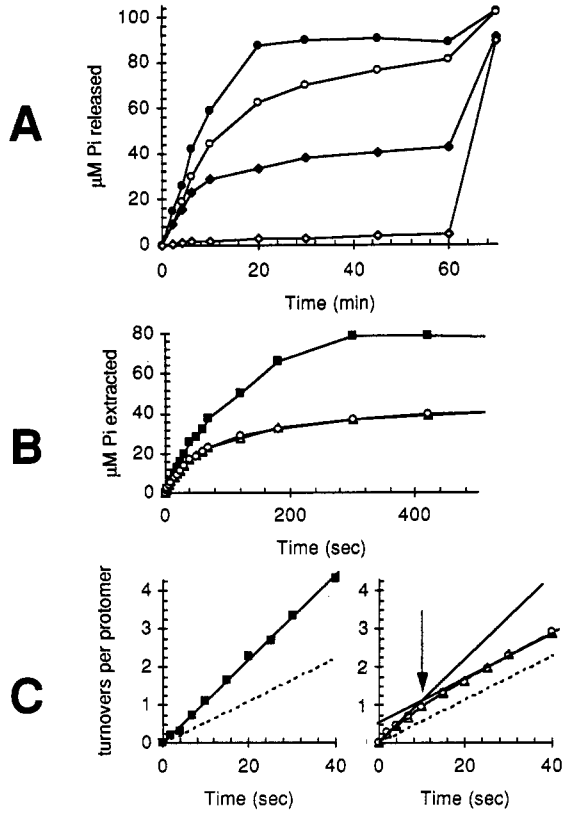


FIGURE 1: Time and K^+ dependence of groES inhibition of the groEL ATPase activity. (A) ATP hydrolysis (25 °C) by 2 μM groEL with 0 (circles) or 3 μM groES (diamonds) was initiated by addition of ATP. Hydrolysis was determined by extracting orthophosphate–molybdate as described in Experimental Procedures. Reaction mixtures contained 1 (open symbols) or 100 mM KCl (closed symbols). At 62 min, 0.2 unit of apyrase (Sigma, grade VIII) was added to 100 μL of each reaction mixture to demonstrate that all remaining counts remained in a hydrolyzable form. (B) Reaction mixtures contained 6 μM groEL, plus 0 (■), 4 (○), or 40 μM groES (△) in standard assay buffer plus 100 mM KCl. (C) The data shown in B was transformed by dividing the concentration of phosphate extracted by the concentration of groEL protomers to give the number of turnovers per groEL protomer. The solid lines were calculated by linear regression analysis of 0–50-s times (groEL alone, left panel) or 10–50-s times (groEL + groES, right panel; intersection marked by arrow) or by fitting to eq 1 (with constants as given in Table I). A dashed line is drawn through the origin with slope equal to the rate of ATP hydrolysis by the groEL/groES complex to illustrate the "burst". The intercept on the *y*-axis corresponding to 0.5 turnovers/groEL protomer is experimentally equivalent to about 8000 dpm of ^{32}P, a value that is clearly distinguishable from the background (~2000 ± 200 dpm).

RESULTS

ATP Hydrolysis by groEL Alone. ATP hydrolysis by groEL alone proceeds linearly, with a k_{cat} (protomer based) of about 5 min^{-1} (Figure 1), until at least 50% of the substrate ATP is consumed. Only thereafter, when the concentration of the product ADP exceeds that of ATP, is there a decrease in the rate of ATP hydrolysis. However, nearly all of the ATP is eventually hydrolyzed. Thus, ADP is only mildly inhibitory to the ATPase activity of groEL alone. Close inspection of the first few turnovers failed to provide evidence for a "burst" of ATP hydrolysis, indicating that product release is not rate-determining (Figure 1C). As previously noted (Gray & Fersht, 1991), the rate of ATP hydrolysis depends on the concentration of MgATP in a cooperative manner.

Figure 2A shows the effect of K^+ concentration on the initial rate of ATP hydrolysis at three concentrations of ATP. In the absence of K^+ the rate of ATP hydrolysis is >10^4-fold less than in the presence of K^+. This is not entirely due to a failure of groEL to bind ATP, since it can be shown that $[groEL]_{14}$ binds at least 4 ATP in the absence of K^+ (Figure 5). In the presence of 50 μM ATP, the ATPase activity is half-maximal at ~0.1 mM K^+ (K_{act}). However, at lower ATP concentrations (*e.g.*, 2 μM ATP), >100 mM K^+ is required. The activation of groEL ATPase by K^+ does not follow simple Michaelis–Menten kinetics at low concentrations of ATP. At 2 μM ATP the K^+ requirement is cooperative with a Hill coefficient of 1.9. The Hill coefficient decreases to ~1 at ATP concentrations higher than 50 μM. Figure 2B further emphasizes the relationship between K_{act} for K^+ and the ATP concentration, demonstrating that, over a 100-fold range of ATP concentration, the K_{act} for KCl varies 1000-fold.

Table I

[groES] (μM)	intercent (turnovers/protomer)	rate (min^{-1}) initial	rate (min^{-1}) final	k_{dec} (min^{-1})
0[a]	0.07 ± 0.09	NA[b]	6.1 ± 0.2	NA
4	0.48 ± 0.08	NA	3.5 ± 0.2	NA
40	0.49 ± 0.12	NA	3.4 ± 0.2	NA
4–40[c]	0.37 ± 0.07	6.1 ● 0.2	3.6 ± 0.7	6.9 ± 0.9

[a] The first three entries in this table are the results of linear regression analysis of the linear portion of the curves in Figure 1C (0–50 s for groEL alone, 10–50 s for groEL/groES). [b] NA, not applicable. [c] The final entry is the result of fitting the groEL/groES data in Figure 1C to eq 1. $[product]_0 = 0$, and the value given in the second column represents the "size of the burst", $(V_i - V_f)/k_{dec}$, for comparison to the linear intercept. Note also that $k_{dec} = k_{cat}$.

Gray and Fersht (1991) showed that the rate of ATP hydrolysis by groEL is cooperative with respect to ATP at 10 mM KCl. However, much higher levels of K^+ are required for maximal activity at low ATP concentrations (Figure 2A) . Figure 2C shows the initial rates of ATP hydrolysis as a function of ATP concentration at three concentrations of K^+. The V_{max} for ATP hydrolysis (5 min^{-1}) is largely independent of the K^+ concentration.[2] However, the $K_{0.5}$ for ATP shows a strong K^+ dependency, decreasing from about 60 μM at 1 mM K^+ to about 5 μM at 0.5 M K^+. The Hill coefficient also depends on the K^+ concentration, increasing from ~2 (500 mM K^+) to ~3 (1 mM K^+). Note that NaCl fails to activate ATP hydrolysis, indicating that the observed changes above are not due to changes in the ionic strength.

ATP Hydrolysis by groEL in the Presence of groES. In the presence of groES the time course of ATP hydrolysis by groEL becomes much more complex (Figure 1). Three distinct phases can be resolved (Scheme I). At the very onset of the reaction (phase I) both toroids turn over at an uninhibited rate (Figure 1C). In phase II, ATP hydrolysis continues at a rate exactly one-half the initial rate (Figure 1B,C). In phase III, a fully inhibited phase, ATP hydrolysis virtually comes to a complete halt (Figure 1A,B). At very low K^+ concentrations, phase II becomes difficult to discern since, under these conditions, phase I proceeds almost immediately to the fully inhibited phase III (Figure 1A).

In phase I the hydrolysis of ATP occurs at an uninhibited rate, regardless of the concentration of groES (Table I). As shown in Figure 1C (arrow), phase II does not occur until 14 mol of ATP is hydrolyzed per mol of $groEL_{14}$. Moreover, extrapolation of the linear portion of phase II to time 0

[2] The specific activity of groEL (turnovers/min) was independent of the groEL concentration (~5 nM to >10 μM) regardless of the potassium concentration, consistent with the observed cooperativity not being caused by dissociation of groEL into smaller subunit assemblies.

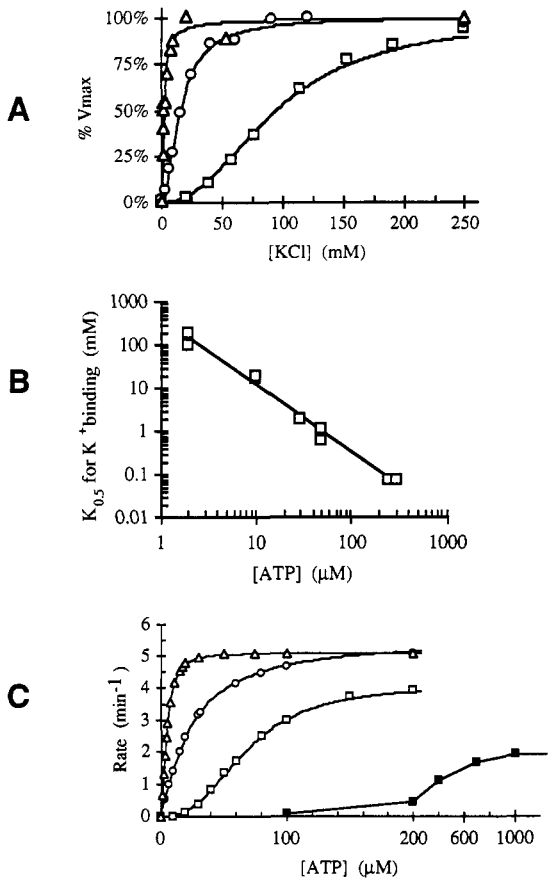


FIGURE 2: Initial velocity of groEL ATP hydrolysis as a function of potassium concentration. (A) Initial rates of [γ-^{32}P]ATP hydrolysis (25 °C) were determined in the standard assay buffer including 2 (□), 10 (○), and 50 μM ATP (△) using 50, 150, and 400 nM groEL, respectively, and the indicated concentrations of KCl. Rates are expressed as percent of the maximum rate of ATP hydrolysis that could be achieved at a particular ATP concentration, which occurred with saturating [KCl]. The lines are drawn using constants obtained by fitting the data to the Hill equation, as described in Data Analyses. The maximum rates were 0.3 min^{-1} at 2 μM ATP, 1.6 min^{-1} at 10 μM ATP, and 4.7 min^{-1} above 50 μM ATP. The Hill coefficient was 1.9 at 2 μM ATP and decreased to ~1 at ATP concentrations greater than 50 μM. (B) Dependence of K_{act} of potassium on ATP concentration. The concentration of potassium required for the half-maximal rate of ATP hydrolysis (K_{act}) is shown as a function of the ATP concentration on a log–log plot in order to emphasize the wide range of concentrations involved. (C) Cooperativity of ATP hydrolysis at three concentrations of K^+. Initial rates of ATP hydrolysis (25 °C) were determined as a function of ATP concentration at three potassium concentrations: 1 (□), 10 (○), and 500 mM (△). Data was fit to the Hill equation, $V_o = V_{max}[S]^n/(K + [S]^n)$, giving V_{max} ~ 4.7 min^{-1} at all potassium concentrations and Hill coefficients of 3, 2.5, and 2.0 at 1, 20, and 500 mM KCl, respectively. Phase II rates of ATP hydrolysis by the groEL/groES binary complex (■) were obtained in standard assay buffer containing 1 mM KCl in the presence of 1 μM groEL, 1 μM groES, 1 mM creatine phosphate, and creatine phosphokinase (0.5 unit/mL). Binary complex (phase III) was first created by adding ATP to 100 μM. After 2 min, the concentration of ATP was raised to the indicated level. Rates of ATP hydrolysis (fraction of complexes in phase II) were then determined as described in Experimental Procedures.

intersects the vertical axis at a point corresponding to turnover at one-half of the sites (7 of 14 protomers, or one of the two toroids) (Table I). As discussed below, ~7 mol of ADP per mol of $groEL_{14}$ in this complex does not readily exchange with free ADP (Figure 3C). Consequently, one half of the sites (corresponding to one of the two toroids) are totally inhibited, while the reaction proceeds at the other seven sites (corresponding to the other toroid) at an uninhibited rate (Scheme I).[3]

The binding of groES to groEL depends upon the presence of an adenine nucleotide (Viitanen et al., 1990). We cannot presently define the kinetic order of events in phase I, *i.e.*, whether the binding of groES occurs before or after the hydrolysis of ATP. In any event, the rate-determining step in the formation of the phase II asymmetric complex is not the binding of groES but rather the hydrolysis of ATP. This is demonstrated by two observations. Firstly, the time course for decay from the uninhibited to the 50% inhibited rate is independent of the concentration of groES (Figure 1C). Secondly, the time course is well described by simply substituting k_{dec}, the rate constant describing the decay from the uninhibited rate to the 50% inhibited rate, with k_{cat}, the rate constant describing the hydrolysis of ATP by groEL (Table I); thus eq 1 becomes

$$\text{no. of turnovers} = [k_{cat}t + (1 - e^{-k_{cat}t})]/2$$

The events associated with the phase I to phase II transition (Scheme I) are further supported by the observation that ~7 mol of [^{32}P]ADP derived from [α-^{32}P]ATP (but not from [γ-^{32}P]ATP) can be trapped in the asymmetric complex in a form that is tightly bound. It does not rapidly exchange with unlabeled ADP (Figure 3C).[4] For example, when the isolated complex, containing [α-^{32}P]ADP, was incubated for 4 h at 25 °C with a 1000-fold molar excess of unlabeled ADP, less than 20% of the radioactivity was lost from the complex (data not shown).

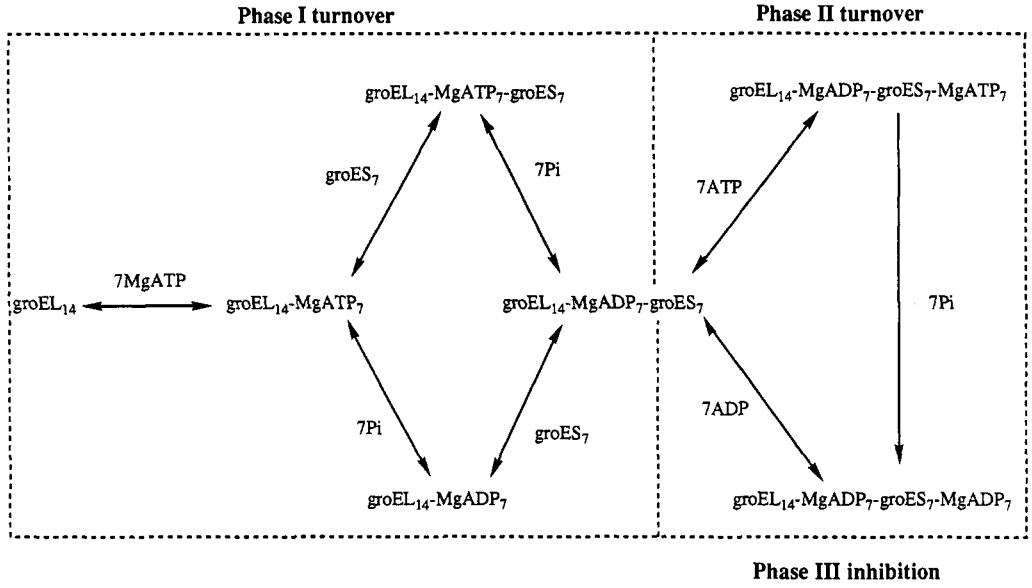
The stoichiometry of $groES_7$ binding to $groEL_{14}$ accompanying the formation of the asymmetric binary complex was determined by two methods (Figures 3). At concentrations of groEL well in excess of the estimated dissociation constant (see below), a stoichiometry of 1.0 $groES_7$ toroid per $groEL_{14}$ double toroid was established, either by measuring the quantity of nonexchangeable ADP bound to $groEL_{14}$ in response to increasing concentrations of $groES_7$ (Figure 3C) or by measuring phase II ATPase activity in response to increasing concentrations of groES (Figure 3A). To determine the dissocation constant for the interaction of groES with groEL, those titrations were repeated at lower concentrations of groEL (Figure 3A), and a value of 26 ± 11 nM, $n = 3$ (eq 2; based on protomers), was obtained, in agreement with Jackson et al. (1993), who reported K_d ~ 3–15 nM.

During phase II the asymmetric chaperonin complex hydrolyzes ATP at one-half the rate with groEL alone (Table I). This has been observed by a number of authors (Chandrasekhar et al., 1986; Grey & Fersht, 1991) who have used a single concentration of K^+, typically 10 mM. However, by varying the concentration of K^+, we have revealed yet another phase of inhibition that is nearly complete. The duration of phase II and the number of turnovers undergone before the fully inhibitory phase III sets in are governed by several factors, of which the concentrations of MgADP, MgATP, and K^+ are the most important. The K^+ ion appears to exert its influence primarily by enhancing the affinity of groEL for ATP (Figure 2). At low concentrations of K^+, where the affinity for MgATP

[3] Caveat: At present we cannot distinguish which of the two $[groEL]_7$ toroids is catalytically active by virtue of containing the nonexchangeable ADP. However, we suspect that the nonexchangeable ADP is bound to the $[groEL]_7$ to which the $[groES]_7$ toroid is also bound. If this is so, the other $[groEL]_7$ toroid is free to engage in nucleotide exchange and ATP hydrolysis.

[4] Experimentally 6 mol of nonexchangeable ADP per mol of $[groEL]_{14}$ is trapped in the asymmetric binary complex rather than 7 (Figure 3). The shortfall can be attributed either to experimental error in the protein concentration or to the presence of inactive groEL. We assume the real stoichiometry is 7 mol of ADP per mol of $[groEL]_{14}$.

Scheme I: Kinetic Phases of ATP Hydrolysis by groEL in the Presence of groES



is least, phase II is of such brief duration that it is impossible to discern since, following phase I (*i.e.*, after at most a single turnover per protomer), the system proceeds almost immediately to the inhibitory phase III (Figures 1A, 3B,C, and 4A,C).

That the phase II to phase III transition is partly due to the system becoming more sensitive to inhibition by ADP can be shown by the inclusion of an ATP-regenerating system (Figure 4B). Under these conditions, phase II is prolonged and ATP hydrolysis continues to completion without the development of the inhibitory phase. Alternatively, enzyme which has entered the inhibitory phase III can be returned to the phase II state (*i.e.*, reactivated) by one of three treatments: (i) If an ATP-regenerating system is added to the inhibited enzyme in phase III (at >5 mM K^+), the hydrolysis of ATP by groEL resumes immediately at the 50%-inhibited, phase II rate and continues to completion (Figure 4B). In this case the system does not revert to the fully inhibited state. (ii) If the concentration of ATP is increased, the enzyme resumes ATP hydrolysis at some fraction of the phase II rate (eq 2) (Figure 2C). The fraction of the phase II rate that is recovered depends on the concentrations of both K^+ and ATP. At 1 mM K^+, the apparent affinity of the remaining sites of the asymmetric binary complex for ATP is ~10-fold reduced compared with that for groEL alone (Figure 2C). (iii) If, after the system has entered the fully inhibited phase III state, the concentration of K^+ is increased, the enzyme resumes ATP hydrolysis at the phase II rate (Figure 4A). However, the reaction does not run to completion but reverts again to the fully inhibited phase III state. But in this instance, the free [ATP]/[ADP] ratio present in the newly established, fully inhibited state at 100 mM K^+ is smaller by a factor of 20 compared with the free [ATP]/[ADP] ratio present in the initial fully inhibited state at 1 mM K^+. This occurs because the K^+ ion increases the affinity of groEL for ATP, such that a larger concentration of ADP must accumulate before complete inhibition occurs. Thus, the catalytically functional toroid in the $groEL_{14}$–$MgADP_7$–$groES_7$ complex, be it the toroid to which $groES_7$ is bound or the other toroid, is sensitized to the free [ATP]/[ADP] ratio quite differently from the toroids in the $groEL_7$–$groEL_7$ complex.

In the presence of low concentrations of K^+ (1 mM) and groES, inhibition of groEL ATPase is complete before a significant portion of the total substrate is hydrolyzed (Figure 1A). Complete inhibition of the ATPase activity (*i.e.*, formation of the phase III state) could be demonstrated upon hydrolysis of as little as 7 μM ATP (Figure 4C) by raising the groEL concentration to 14 μM protomers. Since the product of this reaction, 7 μM ADP, is entirely sequestered as nonexchangeable ADP in the asymmetric complex, phase III inhibition can develop at low concentrations of K^+ without the accumulation of sufficient free ADP to bind at each of the seven remaining sites. In support of this is the observation that at 1 mM K^+ and 100 μM ATP the presence of an ATP-regenerating system neither prevents the development of the phase III state nor reactivates molecules already in the phase III state (Figure 2C). Note that groEL alone turns over in the presence of 1 mM K^+ and 90 μM ATP and in the absence of groES at about 3 min^{-1} (Figure 2C). Yet under otherwise identical conditions the asymmetric binary complex is essentially unable to hydrolyze ATP (Figures 1A and 2C).

Since groES could inhibit completely groEL with as little as one ring of seven subunits having turned over, we attempted to form the complex starting from free ADP. Figure 4C shows the amount of newly hydrolyzed ATP needed to inhibit groEL after 2 min of incubation with 20, 100, and 250 μM ADP. As the concentration of ADP is increased, progressively fewer turnovers are required to form the fully inhibited phase III state, indicating that the asymmetric chaperonin complex can be formed by starting from free ADP. However, since the affinity of groEL alone for ADP is not strong, considerably greater concentrations of free ADP (or longer times) are required to form the complex than when the ADP is generated in situ from ATP.

ATP Binding to groEL. The ability of groEL to bind ATP in the absence of hydrolysis was tested using gel filtration under equilibrium conditions (Hummel & Dreyer, 1962). Using a P-2 column equilibrated with [γ-^{32}P]ATP, binding is manifest as an increase in ligand co-eluting with the protein and an *equivalent* decrease in ligand eluting with the total bed volume. Figure 5A demonstrates equilibrium binding of ATP to groEL in the absence of K^+. Note the presence of a plateau between the peak and the trough, indicating that the system is at equilibrium. The amount of nucleotide bound to groEL was quantitated both by the increase in ligand co-eluting with groEL and by the corresponding decrease in ligand eluting at the column's total bed volume (Figure 5B). These values were ~4 ATP/$[groEL]_{14}$ when the column was equilibrated

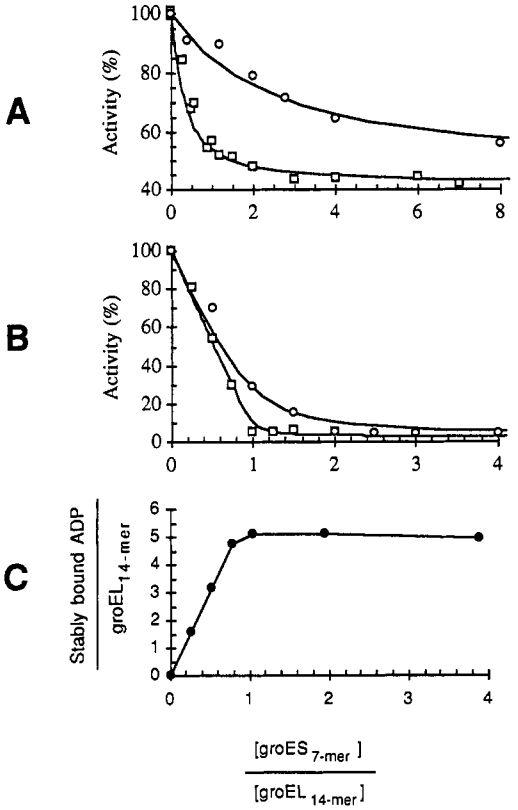


FIGURE 3: Inhibition of groEL ATPase activity by groES. (A) Rates of ATP hydrolysis (25 °C) were obtained at 1.0 μM (□) or 50 nM groEL (○) in standard assay buffer with 100 μM ATP, 100 mM KCl, and increasing concentrations of groES. The percent of ATPase activity remaining (relative to the rate of ATP hydrolysis in the absence of groES) is shown as a function of increasing the ratio of $groES_7$ to $groEL_{14}$. Lines were drawn using eq 2 as described in Data Analyses. The rates measured involved multiple turnovers of groEL so that the contribution of the first, phase I turnover can be ignored. (B) Fully inhibited rates of ATP hydrolysis were obtained as in (A), using 1.0 μM (□) or 100 nM groEL (○), in buffer containing 1 mM KCl. (C) Quantitation of stably bound nucleotide in the groEL/groES complex. Reaction mixtures containing 2.2 μM $groEL_{14}$ and the indicated concentrations of $groES_7$ in 50 mM Tris-HCl, pH 7.8, 0.05 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, and 0.5 mM KCl were made 100 μM in [α-^{32}P]ATP (0.045 Ci mmol^{-1}). After 1 min, 100 μL was injected onto a Sephadex G-50 column equilibrated with the same buffer lacking ATP but including 50 μM ADP. One-half-milliliter fractions were collected, and 200-μL aliquots were counted to determine the location of nucleotide. GroEL, groES, and the groEL–groES complex elute together at 5.5–6.0 mL, well separated from unbound labeled nucleotde.

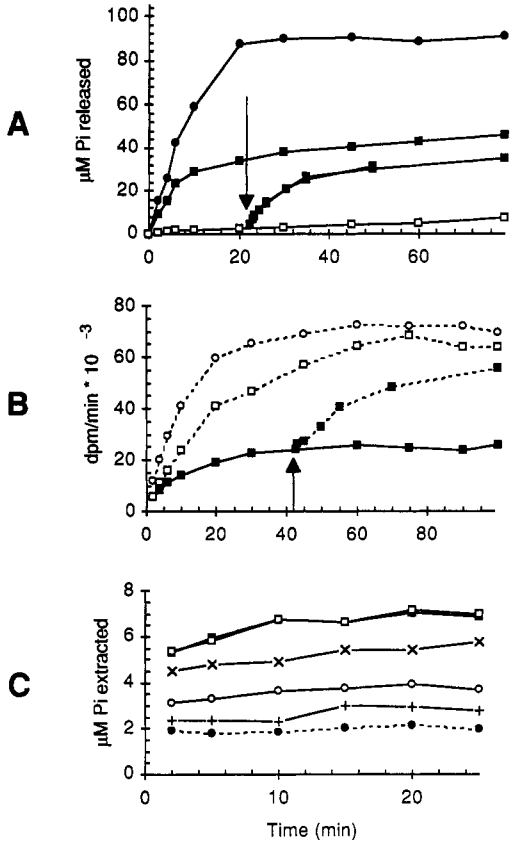


FIGURE 4: (A) Raising the potassium concentration restarts the groEL ATPase. Reaction mixtures contained standard assay buffer plus 1 (open symbols) or 100 mM KCl (closed symbols) and 2 μM groEL plus 0 μM (circles) or 3 μM groES (squares). At 22.5 min (arrow) a portion of the 1 mM KCl reaction mixture containing both groEL and groES was adjusted to 100 mM KCl. (B) Removal of ADP restarts the groEL ATPase. ATPase reactions contained standard assay buffer with 5 mM KCl, 100 μM ATP, and 2 μM groEL with 0 (circles) or 3 μM groES (squares). Creatine phosphate (2.5 mM) and creatine phosphokinase (2.5 units/mL) were either absent (solid line) or added at 0 or 42 min as indicated (dashed lines). Dpm/min are shown, as the specific activity of the ATP in the presence of CP/CPK was diluted with time. (C) Turnovers before complete inhibition of groEL by groES; ATPase assays in standard assay buffer containing 1 mM KCl, 100 μM ATP, 14 μM groEL, 9 μM groES, and various concentrations of ADP. GroEL and groES were incubated with 0 (□), 20 (×), 100 (○), or 250 μM ADP (+) for 2 min or 100 μM ADP for 60 min (●) before reactions were started by addition of ATP to 100 μM.

with either 45 or 90 μM ATP. It could be shown by assessing each fraction for $^{32}P_i$ that no hydrolysis had occurred during gel filtration (Figure 5B).

To emphasize the strict K^+ requirement for groEL ATP hydrolysis, similar experiments were done in the presence of 1 mM KCl (Figure 5A). Under these conditions there was no clearly defined plateau between the peak and the trough, indicating a system not at equilibrium. Thus, no meaningful values for ATP binding to groEL can be obtained in the presence of K^+.

Under equilibrium conditions, ATP *can* bind to groEL in the absence of ATP hydrolysis and *at least* four nucleotides can bind per $groEL_{14}$ (in the absence of K^+). For several reasons all 14 ATP binding sites may not be populated with ATP. For example, in the absence of K^+, the affinity of the remaining sites may be so weak as to preclude complete site occupancy. The decrease in the $K_{0.5}$ for ATP, measured kinetically (Figure 2C), is consistent with this explanation. Alternatively, the binding of a full complement of nucleotide may require a longer time to reach equilibrium.

DISCUSSION

In the absence of groES, the interaction of groEL with both K^+ and ATP is cooperative and synergistic. High concentrations of either ligand increase the apparent affinity of groEL for the other ligand, but decrease the degree of cooperativity (Hill number) associated with that ligand.

In the presence of groES the time course of ATP hydrolysis is more complex. Two transitions are apparent. The first involves the conversion of $groEL_{14}$ to an asymmetric complex, $groEL_{14}$–$MgADP_7$–$groES_7$, which retains half of the sites in an active or potentially active form (Scheme I). The rate at which this transition occurs is determined by the rate of ATP hydrolysis (Figure 1C; Table I) and involves only half of the sites on groEL (Figures 1C and 3A). A single toroid of $groES_7$ per $groEL_{14}$ double toroid is sufficient to cause the transition (Figure 3), and ~7 mol of [α-^{32}P]ADP, derived from [α-^{32}P]ATP, is trapped in a nonexchangeable form as a result.

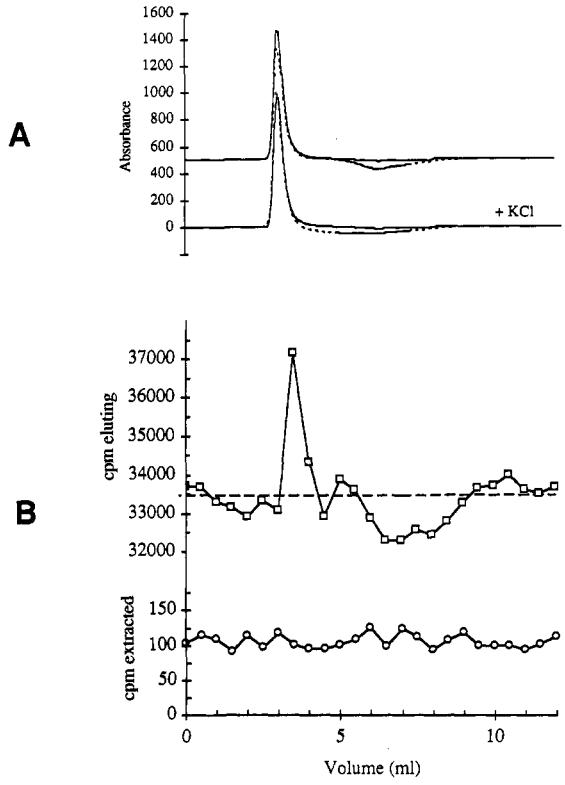


FIGURE 5: GroEL binds ATP in the absence of potassium. (A) One hundred microliters of 218 μM groEL was applied to a P-2 column (1.0 × 12 cm, Bio-Rad 200–400 mesh) equilibrated with 100 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol, containing 45 μM [γ-^{32}P]ATP (upper tracings), or the same buffer with 1 mM KCl (lower tracings); absorbance at 260 (dashed line) and 280 nm (solid line) was monitored (HP1050 multiwavelength detector). The lack of a plateau between the peak and the trough when potassium is present confirms that the system is not at equilibrium. (B) Fifty-microliter aliquots of each 0.50-mL fraction were counted (□); orthophosphate–molybdate was extracted from 25-μl aliquots (as described in Experimental Procedures) and counted (○) to determine the amount of ATP hydrolyzed. Cpm shown are not corrected for ^{32}P background of ~0.7% (100 cpm).

The recent data of Jackson et al. (1993) demonstrate that the initial rate of ATPase hydrolysis by the groEL/groES complex lacks the burst. The reason for this discrepency is unclear; however, the difference may be related to the method of quenching the ATPase activity prior to extraction of P_i.

Electron micrographs of these and related complexes clearly show asymmetric, bullet-shaped images, consistent with $groES_7$ interacting with only one of the two potential end-surfaces of $groEL_7$–$groEL_7$ (Saibil et al., 1991; Ishii et al., 1992; Langer et al., 1992). The chaperonins from *Thermus thermophilus* are copurified as an asymmetric binary complex which contains tightly bound ADP (Yoshida et al., 1993). The binding of groES to groEL during this first transition presumably involves a conformational change in groEL. The binding and hydrolysis of ATP, or less favorably, of ADP (Figure 4C), most probably triggers this change.

The second transition, from the 50%-inhibited, phase II state to the fully inhibited, phase III state is more perplexing. One possible explanation for the phase II to phase III transition would be the interaction of a second toroid of $groES_7$ with the remaining free heptameric $groEL_7$ toroid of the asymmetric complex, so as to create a symmetrical, fully inhibited [groEL–ADP–groES]$_7$–[groEL–ADP–groES]$_7$ complex. Indeed, some symmetrical "football-shaped"[5] images of groEL and groES in the presence of ATP have been observed by electron microscopy (M. Schmid, personal communication). However, the stoichiometry of nonexchangeable ADP bound to the fully inhibited phase III complex remains 0.5 mol per mol of groEL protomer, even in the presence of a 4-fold molar excess of groES over groEL (Figure 3). Moreover, a single $groES_7$ toroid is sufficient to cause the doubly toroidal [groEL]$_7$–[groEL]$_7$ to enter the phase III state (Figure 3B,C). An alternative explanation for the phase II to phase III transition involves a large increase in the affinity of the remaining sites for ADP. The ability of added ADP to reduce the number of turnovers undergone by groEL prior to the onset of complete inhibition (Figure 4C) and the ability of an ATP-regenerating system to relieve or prevent the development of phase III inhibition are consistent with this explanation. However, this cannot be the sole cause of the transition. Firstly, initial studies of the equilibrium binding of ADP to groEL alone and to the free sites of the asymmetric binary complex indicate only small differences in affinity (M. J. Todd, unpublished data). Secondly, at low K^+ concentrations it is possible to create a fully inhibited complex after turning over one-half of the sites (Figure 4C), *i.e.*, when there is insufficient ADP to inhibit the remaining sites. Thirdly, addition of an ATP-regenerating system at low K^+ fails to relieve or prevent the development of phase III inhibition. Instead, the phase II to phase III transition can mostly be attributed to a large decrease in the affinity of the remaining sites in the asymmetric binary complex for ATP (Figure 2C). This is particularly apparent when measurements are made under conditions where the phase II to phase III transition is most evident: at low concentrations of K^+ (Figures 1A, 2C, 3B, and 4A,C).

The kinetics of ATP hydrolysis by the chaperonins represent a remarkable combination of three kinetic phenomena, which, considered individually, have been previously described. The first concerns the cooperative and synergistic interactions between ligands, such as MgATP and K^+, a phenomenon first recorded 70 years ago (Hill, 1925) and often observed with oligomeric proteins. This cooperativity can be explained in terms of the heterologous interactions of the individual subunits within a single toroid (intratoroidal communication). The second phenomenon, tight-binding inhibition of $groEL_{14}$ ATPase activity by $groES_7$, is similar in nature to the interaction of several enzymes with small-molecule inhibitors, with the complication that only half of the sites react in this manner. The third phenomenon, half-of-the-sites reactivity, has also been previously described (Fersht, 1985) and is evidently imposed upon the doubly toroidal $groEL_{14}$ by interaction with $groES_7$. However, the kinetic properties of the remaining sites are altered, implying intertoroidal communication. This behavior can be explained on the basis of the isologous interactions of the individual subunits between different toroids. To the best of our knowledge, these three phenomena have not been previously reported in a single system. The manner in which these properties are exploited to bring about the release of target proteins remains to be determined.

[5] We refer here to American football and not soccer.

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