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Asymmetry, Commitment and Inhibition in the GroE ATPase Cycle Impose Alternating Functions on the Two GroEL Rings

Neil M. Kad*, Neil A. Ranson, Matthew J. Cliff and Anthony R. Clarke

Department of Biochemistry School of Medical Sciences University Walk, University of Bristol, Bristol BS8 1TD, UK

The ATPase cycle of GroE chaperonins has been examined by transient kinetics to dissect partial reactions in complexes where GroEL is asymmetrically loaded with nucleotides. The occupation of one heptameric ring by ADP does not inhibit the loading of the other with ATP nor does it prevent the consequent structural rearrangement to the ''open'' state. However, ADP binding completely inhibits ATP hydrolysis in the asymmetric complex, i.e. ATP cannot by hydrolysed when ADP is bound to the other ring. This non-competitive inhibition of the ATPase by ADP is consistent with a ring-switching, or ''two-stroke'', mechanism of the type: ATP:GroEL → ADP:GroEL → ADP:GroEL:ATP → GroEL:ATP → GroEL:ADP, i.e. with respect to the GroEL rings, ATP turns over in an alternating fashion. When the ATP-stabilized, ''open'' state is challenged with hexokinase and glucose, to quench the free ATP, the open state relaxes slowly (0.44 s^{-1}) back to the apo (or closed) conformation. This rate, however, is three times faster than the hydrolytic step, showing that bound ATP is not committed to hydrolysis. When GroES is bound to the GroEL:ATP complex and the system is quenched in the same way, approximately half of the bound ATP undergoes hydrolysis on the chaperonin complex showing that the co-protein increases the degree of commitment. Thus, non-competitive inhibition of ATP hydrolysis, combined with the ability of the co-protein to block ligand exchange between rings has the effect of imposing a reciprocating cycle of reactions with ATP hydrolysing, and GroES binding, on each of the GroEL rings in turn. Taken together, these data imply that the dominant, productive steady state reaction *in vivo* is: GroEL:ATP:GroES → GroEL:ADP:GroES → ATP:GroEL:ADP:GroES → ATP:GroEL:ADP → GroES:ATP:GroEL:ADP → GroES:ATP:GroEL for a hemi-cycle, and that significant inhibition of hydrolysis may arise through the formation of a dead-end ADP:GroEL:ATP:GroES complex.

Keywords: GroE; chaperonin; transient kinetics; non-competitive inhibition; protein folding

*Corresponding author

Introduction

The *Escherichia coli* chaperonins, GroEL and GroES, are the most widely studied members of a family of proteins which interact with non-native proteins during their folding reactions, and are required for cell viability at all temperatures *in vivo* (Fayet *et al.*, 1989). Together, they can increase the efficiency, and sometimes the rate, on the *in vitro* folding of certain proteins in an ATP-dependent manner (Goloubinoff *et al.*, 1989; Martin *et al.*, 1991, 1993; Weissman *et al.*, 1994; Fisher, 1994; Ranson *et al.*, 1995). GroEL is a tetradecamer of identical 57 kDa subunits (Hemmingsen *et al.*, 1988) which forms two heptameric rings (Saibil *et al.*, 1991; Braig *et al.*, 1994). The atomic structure of GroEL shows that each subunit consists of three domains: an equatorial domain which forms the core of the GroEL complex when the two rings stack in a back-to-back fashion, an apical domain which

Present address: N. A. Ranson, Department of Crystallography, Birkbeck College London, Malet Street, London WC1E 7HX, UK.

Abbreviations used: TEA, triethanolamine hydrochloride; PMSF, phenylmethylsulphonyl fluoride.

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forms the ends of the chaperonin complex, and a smaller intermediate domain which links the two (Braig *et al*., 1994). The co-protein, GroES, a single heptameric ring of identical 10.4 kDa subunits (Chandrasekhar *et al*., 1986; Hunt *et al*., 1996), binds to one or both ends of the GroEL oligomer in the presence of adenine nucleotides (Saibil *et al*., 1991; Chen *et al*., 1994; Llorca *et al*., 1994; Schmidt *et al*., 1994; Azem *et al*., 1995; Roseman *et al*., 1996; Xu *et al*., 1997).

The hierarchical, oligomeric structure of GroEL allows the binding of nucleotides to be regulated by a complex system of co-operativity. ATP binds with strong, positive co-operativity (Gray & Fersht, 1991; Bochkareva *et al*., 1992; Jackson *et al*., 1993). However, more recently it has been established that this co-operative binding event involves the binding of only seven ATP molecules to one of the two available GroEL rings. A second level of co-operativity exists to reduce the affinity of the second ring for ATP once the first ring has been occupied (Yifrach & Horovitz, 1994, 1995; Burston *et al*., 1995). This negative co-operativity requires communication between the rings which is propagated *via* the two inter-ring, equatorial domain contacts which anchor the GroEL complex together (Roseman *et al*., 1996). This has the effect of stabilizing asymmetrical structures (i.e. GroEL complexes with different nucleotides bound to each ring) and destabilizing symmetrical complexes (i.e. GroEL complexes with the same nucleotide bound to each ring). The conformational changes responsible for these co-operative effects are large, and have been visualized by cryo-electron microscopy (Roseman *et al*., 1996) and more recently by X-ray crystallography (Xu *et al*., 1997). ATP binding induces a large opening and rotation in the apical domains of GroEL complexes; these rearrangements occur about the flexible hinge-like intermediate domain, and re-orientate the polypeptide binding sites away from the central cavity. These structural data are in precise agreement with biochemical evidence showing that binding of ATP reduces the affinity of a GroEL ring for substrate proteins (Badcoe *et al*., 1991; Jackson *et al*., 1993; Staniforth *et al*., 1994; Todd *et al*., 1994).

Much progress has been made in elucidating how this basic switching of affinity for non-native polypeptides is used by the chaperonins to increase the efficiency of protein folding (Jackson *et al*., 1993; Weissman *et al*., 1995, 1996; Mayhew *et al*., 1996; Ranson *et al*.,1997). Such models envisage the initial binding of the substrate protein to the uncapped ring of a GroEL:GroES complex to form a *trans* complex, i.e. in which GroES and the substrate protein are located at opposite ends of the GroEL oligomer (Weissman *et al*., 1995). Subsequent release and rebinding of GroES to the other ring occurs once per ATPase cycle (Burston *et al*., 1995) and leads to the formation of a *cis* complex with the substrate protein encapsulated under GroES. In this complex the substrate, although caged, is displaced from the binding surfaces of GroEL and is free to fold before being ejected into bulk solution on the next round of hydrolysis. The chance of a monomeric substrate attaining the native state (Weissman *et al*., 1995, 1996; Mayhew *et al*., 1996), or a subunit of a multimeric protein being committed to productive assembly during a single visit, is governed by a balance between the dwell time in the cavity and the unimolecular time constant for the committed folding step (Ranson *et al*., 1997; Rye *et al*., 1997). Hence, whether folded or not, substrate proteins are released from the GroEL complex on each round of hydrolysis. If released in an uncommitted state, substrates can rebind to GroEL for another round of chaperonin-assisted folding (Weissman *et al*., 1994; Taguchi & Yoshida, 1995; Burston *et al*., 1996; Ranson *et al*., 1997).

From the above description, it is evident that current models envisage a side-to-side change in the protein-binding affinity of GroEL (Todd *et al*., 1994; Burston *et al*., 1995) yet there remains no clear kinetic evidence of such a reciprocating mechanism in the ATPase cycle of the GroE system. The fact that we do not fully understand the mechanism is not entirely surprising since, formally, the system consists of at least 25 potential states (see Figure 1). Thus, the reaction cycle cannot be said to be fully defined until the preferred pathway(s) through these states have been mapped. For a 25-state system, the number of possible pathways is alarmingly large, and it must be appreciated that the relative energies and thus the populations of these states will change according to the concentration of free nucleotide, i.e. the relative proportion of GroEL in each state is dependent upon the prevailing conditions. The 25-state model does not include the initial products of hydrolysis, ADP:Pi:GroEL or GroES:ADP:Pi:GroEL as the release of phosphate from the complex is coincident with ATP cleavage (S. G. Burston, M. Webb & A.R.C., unpublished results).

Here we examine firstly the inner states in the global model (i.e. those states in Figure 1 which arise in the absence of GroES) by isolating the given steps in single-turnover conditions. We then attempt to expand to the outer complexes by considering the influences of GroES on the system.

Results and Discussion

In the absence of GroES, the global scheme shown in Figure 1(a) and (b) simplifies to that shown in Figure 2(a), and a considerable body of experimental evidence exists to describe the way in which these states behave. Positive co-operativity (Gray & Fersht, 1991; Bochkareva & Girshovich, 1994; Burston *et al*., 1995) serves to promote the tight binding of ATP to one ring of GroEL, but negative cooperativity between the two GroEL rings results in weaker binding of ATP to the second ring (Yifrach & Horovitz, 1994, 1995; Bochkareva & Girshovich, 1994; Burston *et al*.,

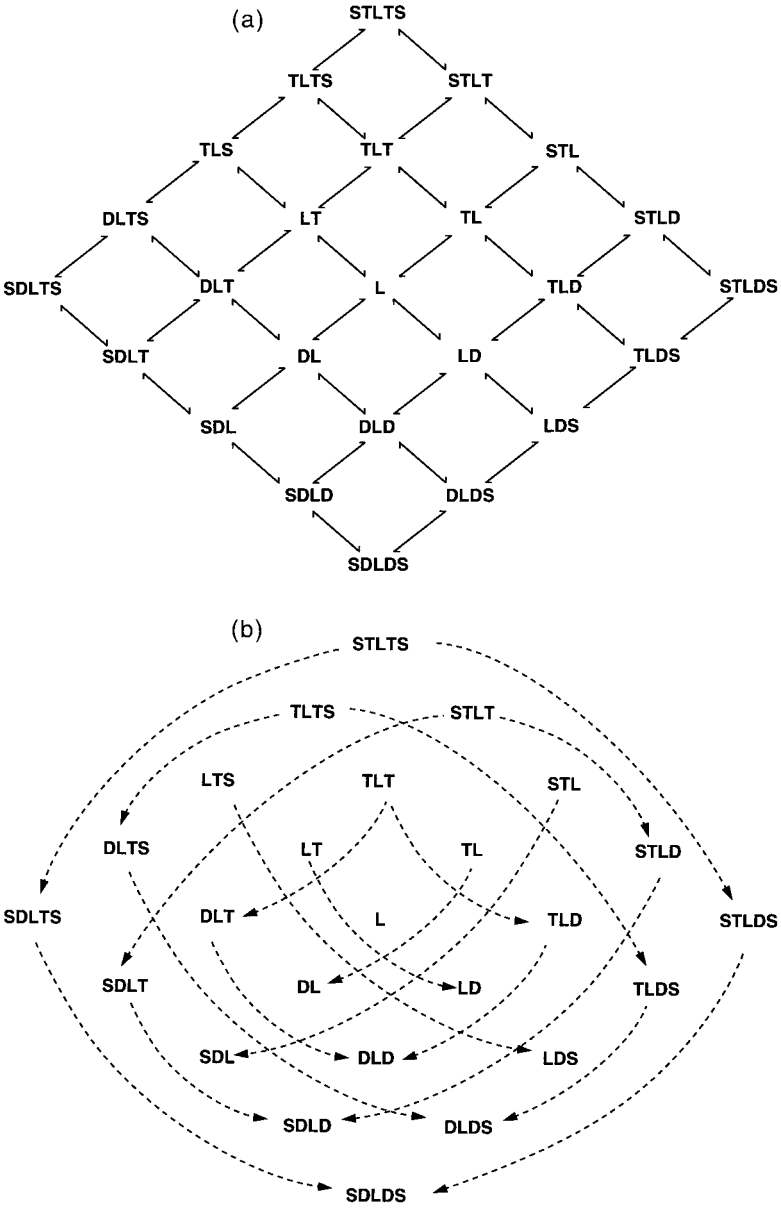

Figure 1. Global models for the interactions of GroEL, GroES and nucleotides. L and S denote GroEL and GroES oligomers, respectively, while T and D refer to GroEL rings occupied with ATP and ADP, respectively. Asymmetrically related complexes are distinguished by the orientation of nucleotide (and GroES) relative to L; thus, SDL and LDS are structurally identical states with nucleotide and co-protein occupying opposite GroEL rings. Shown in (a) are all possible states that can arise from the interaction of these four components. The same model is presented in (b) with the possible conversions between states owing the hydrolysis of bound ATP. It is emphasized that these models are presented to show the complexity of the system and contain no pathways or suggested mechanism. ADP:Pi states are omitted as phosphate release is coincident with hydrolysis (see the Introduction).

1995). This is taken to mean that the asymmetric states (TL, LT, DL, LD, TLD and DLT) are more favoured than the symmetrical TLT and DLD states.

In a previous study (Burston *et al.*, 1995) it was suggested that TLD or DLT could undergo hydrolysis to form DLD and that ADP dissociation may occur from the opposite ring to give DL or LD, respectively. Although such a mechanism would result in an alternating mechanism (TLD → DLD → DL etc.), there is no direct evidence to confirm the validity of the model, since no practical means of kinetically isolating the two rings has yet been achieved. In view of results with show that ADP is a steady-state inhibitor of the GroEL ATPase (Bochkareva & Girshovich, 1994; Todd *et al.*, 1994), we consider here a more direct mechanism which may promote end-to-end switching. The mechanism is predicted on two properties. The first is already established, that negative co-operativity makes the symmetrical TLT complex difficult to form. The second is open to experiment; namely that if ADP acts as a strict non-competitive inhibitor of the ATPase activity on the opposite ring, then TLD (or DLT) would be hydrolytically dead. It is evident from Figure 2(b) that a combination of these factors would predispose the system to alternate the ATPase reaction between rings.

Optical signals for ATP binding and hydrolysis

To examine the above possibility, we exploited the well-established changes in fluorescence signal when ATP binds to pyrenyl-GroEL (Jackson *et al.*, 1993; Burston *et al.*, 1995). As shown in Figure 3(a) (heavy line), when a high concentration of the GroEL oligomer is challenged with seven equiva-

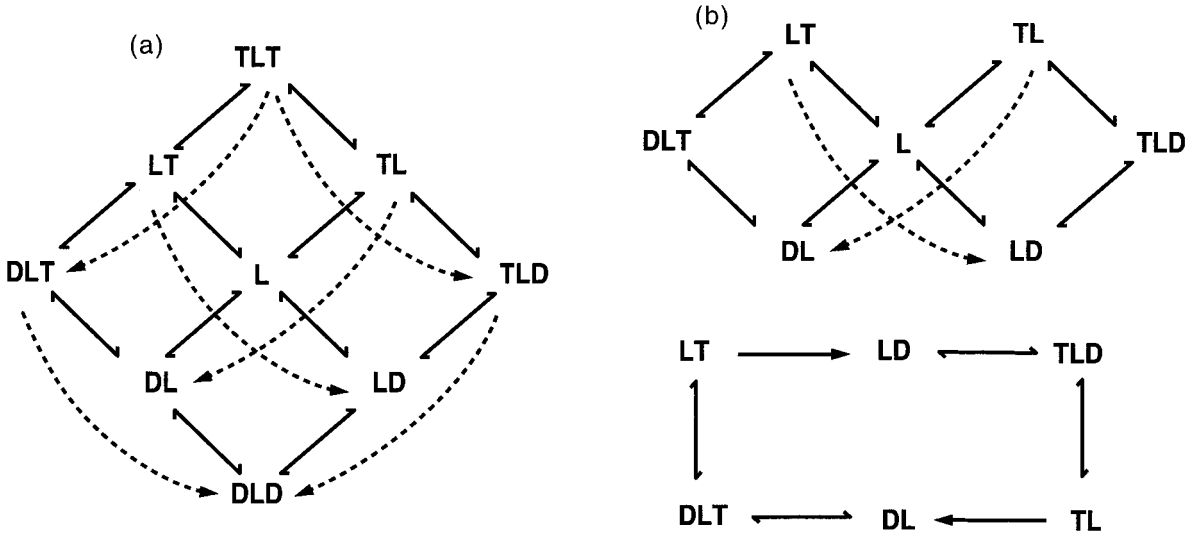

Figure 2. Possible mechanisms to enforce hydrolysis of ATP on alternating rings of the GroEL oligomer. (a) A mechanism for the GroEL ATPase cycle previously proposed as the simplest model to fit the available data (Burston *et al.*, 1995). The inner states of the global system (i.e. those with no GroES) described in Figure 1 are shown here. Hydrolysis can occur in an asymmetric complex (TLD or DLT) to form a symmetric complex (DLD) and hydrolysis on one ring was proposed to promote dissociation of ADP from the other, although no direct evidence for this step was available. (b) A more simple method of enforcing end-to-end switching, as inhibition of hydrolysis in the asymmetric complexes enforces ring-switching. If ADP inhibits hydrolysis in the asymmetric states (DLT and TLD) it is then necessary for this ADP to dissociate before the cycle can continue. Once dissociated, hydrolysis of ATP proceeds to form DL and LD, the empty ring now binds ATP and the cycle is reversed; therefore through a simple mechanism of ADP inhibition ring-switching is enforced.

lents of ATP there is an enhancement of fluorescence as the occupied ring is converted to the open state (also called the R-state), followed by a decrease in fluorescence as the nucleotide is hydrolysed. Both processes are exponential; the first occurring at approximately 2 s^{-1}, and the second at approximately 0.15 s^{-1}, the rate of ATP hydrolysis. The conditions for this experiment were chosen such that the concentration of GroEL subunits present is much greater than the concentration of ATP required for half saturation of GroEL (i.e. $E_o \gg K_{0.5}$) and thus conform to tight binding conditions, i.e.

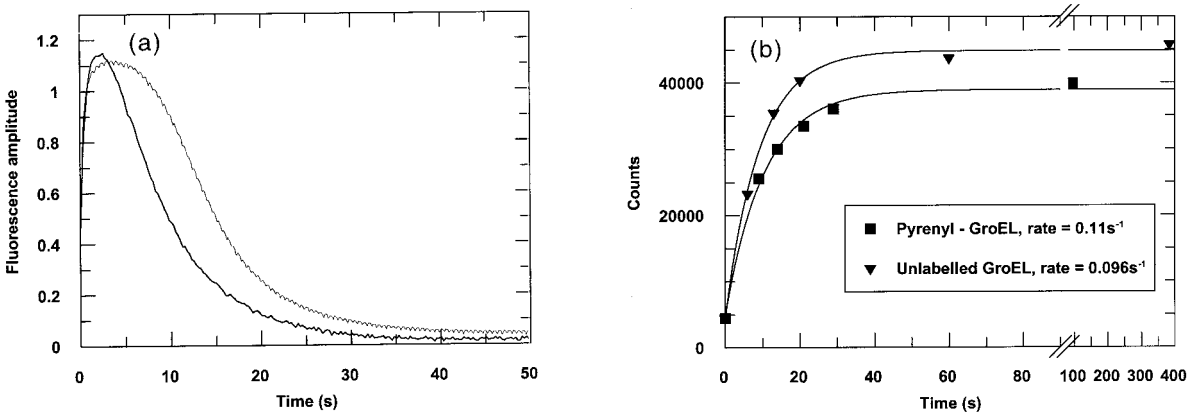

Figure 3. Comparison of single and double turnover transients upon addition of ATP to pyrenyl:GroEL by (a) pyrene fluorescence, and (b) ATP hydrolytic assay. (a) The 360 μM pyrenyl-GroEL (subunit concentration) in the standard buffer, was mixed with either 180 μM or 360 μM ATP in a stopped-flow apparatus such that the final post-mix concentrations were 180 μM pyrenyl:GroEL and either 90 μM (heavy line) or 180 μM (light line) ATP, corresponding to a single and double turnover of the GroEL ATPase, respectively. The reaction was monitored by exciting at 343 nm and collecting all light emitted with a wavelength greater than 360 nm. When fit to two consecutive exponentials the rates obtained are 2.0 (±0.065) s^{-1} for the up-phase, and 0.145 (±0.001) s^{-1} for hydrolysis; and for the double turnover 2.48 (±0.13) s^{-1} and 0.1523 (±0.0006) s^{-1}, respectively. (b) 276 μM of either labelled (■) or unlabelled (▼) GroEL subunits were mixed with ATP such that the final concentration of ATP was 100 μM and GroEL 230 μM. Aliquots (20 μl) were withdrawn at the given times before being acid-quenched and the extent of ATP hydrolysis quantified as described in Materials and Methods. Both traces were fit to a single exponential equation and provided similar rate constants 0.109 (±0.0063) s^{-1} (unlabelled) and 0.096 (±0.009) s^{-1} (labelled).

there is a negligible amount of free ATP during turnover. The decay in fluorescence observed is more complex if concentrations of ATP greater than half of the available GroEL subunits are used (Figure 3(a), light line); the single exponential decay in fluorescence is preceded by a lag phase which corresponds to the steady-state hydrolysis of ATP before the final single turnover, which occurs at the same rate as described above.

To ensure that the above assignments of optical signals are valid and allow the interpretation of stopped-flow data presented hereafter, the hydrolytic rate of the pyrenyl:GroEL complex under these conditions was directly measured. Shown in Figure 3(b) are the single turnover transient kinetics of ATP hydrolysis by unlabelled and pyrenyl:-GroEL. Both reaction transients are single-exponential processes, which optimally fit to observed rate constants (k_{obs}) of 0.11 s^{-1} and 0.10 s^{-1}, respectively. These rates are consistent with those measured previously (Burston *et al.*, 1995), and with the rate of the slow fluorescence decrease in stopped-flow experiments (Figure 3(a)), and demonstrate that pyrenyl:GroEL behaves in an essentially identical manner to unlabelled GroEL with respect to ATP hydrolysis.

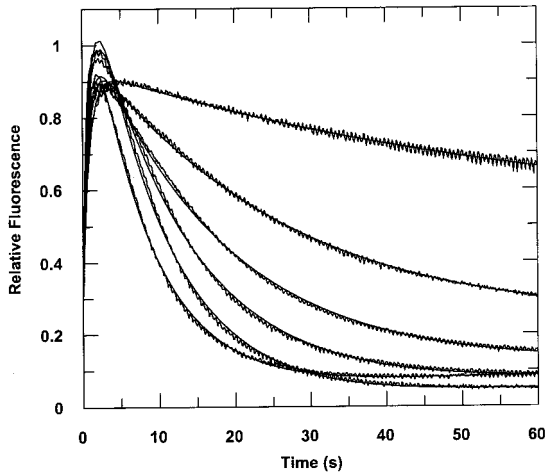

Figure 4. The effect of ADP on the single turnover kinetics of pyrenyl:GroEL. Pyrenyl:GroEL was rapidly mixed in a stopped-flow fluorimeter with ATP and ADP, yielding a final concentration of 95 μM GroEL (active subunits) and 9 μM ATP and the given concentrations of inhibitory ADP. The traces show two phases; a rapid rise in fluorescence corresponding to a conformational rearrangement caused by the binding of ATP, and a slower reduction in fluorescence corresponding to hydrolysis of ATP. ADP concentrations used (from bottom to top trace) were 0, 100, 250, 500, 750, 1000, 2000 and 10,000 μM ADP, and it is clearly shown that ADP inhibits the rate of hydrolysis. The traces were fit to the analytical solution for two sequential irreversible processes; the results are shown in Figures 5 and 6.

The influence of ADP on the GroEL ATPase

Having secured the interpretation of the signal, the use of such transient kinetic techniques is ideal for examining the behaviour of mixed ADP:ATP complexes for three reasons. Firstly, determining the nature of inhibition (whether it is competitive, uncompetitive or mixed) by use of steady-state kinetics in a system which shows both positive and negative co-operativity, is dauntingly complicated. Secondly, using the transient method allows us to dissect ATP binding, and the resultant structural rearrangement, from the following hydrolysis of the nucleotide. Thirdly, the rates of these exponential reactions can be measured with great accuracy.

The effect of ADP on the observed reaction transients is shown in Figures 4 and 5. The former plot shows that the observed rate of the hydrolytic single-turnover reaction progressively decreases as the concentration of ADP is increased. In contrast, the rate of the ATP-induced conformational change (Figure 5, and inset) is only slightly influenced by the inclusion of ADP in the reaction. Neither reaction phase is influenced by the order of mixing in the stopped-flow apparatus (data not shown).

The plots presented in Figure 6 show the effect of ADP on the observed rate constant for ATP hydrolysis. In these experiments the ADP concentration was varied at a series of fixed ATP concentrations and the inset to the Figure shows a secondary plot of the apparent inhibition constant (K_i) as a function of the ATP concentration. Two conclusions can be drawn from these results. Firstly, at infinite ADP concentrations, hydrolysis of ATP is completely abolished. Secondly, the apparent inhibition constant (~0.7 mM) does not increase as the concentration of ATP is raised (see inset to Figure 6). The corollary is that ADP acts as a non-competitive inhibitor of the ATPase reaction and that the ATP_7:$GroEL_{14}$:ADP_7 complex (TLD) is completely unable to undergo hydrolysis. Hence ADP blocks the cleavage of ATP but not its binding.

Returning to the model shown in Figure 2(a), we can now show that TLD and DLT must first undergo ADP dissociation before ATP hydrolysis. At first sight, such a mechanism enforces hydrolysis (and the accompanying switch in protein binding affinity) on each of the GroEL rings in an alternating fashion. However, this cycle as proposed is not sufficient to define the GroEL ATPase reaction fully. The possibility remains that the nucleotide in the asymmetric nucleotide states (with either ATP (TL and LT), or ADP (DL and LD) bound to one of the GroEL rings) can dissociate and re-associate to the opposite ring. This would lead to the reaction cycle randomizing and hydrolysis proceeding on either ring, rather than in the side-to-side manner discussed above. In such a case, the flux through the cycle would be defined by the rates of dissociation of ADP and ATP from a GroEL ring, and the hydrolytic rate.

It has been reported that the binding of GroES to a GroEL:ATP ring (i.e. the step TL + S → STL and its asymmetric partner) commits the bound ATP to

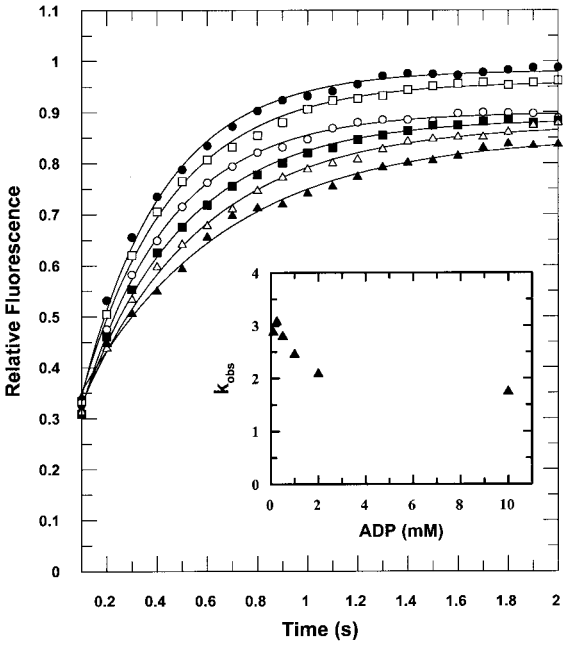

Figure 5. ADP has no effect on the binding-induced conformational change. This plot is a magnification of the first phase shown in Figure 4. The rate of conformational change can be seen not to vary significantly over the range of ADP concentrations used, implying ADP has no effect on the binding of nucleotide. The traces were fitted to single exponentials in order to give a more accurate estimation of the observed rate; the inset is a direct plot of rate variation with ADP.

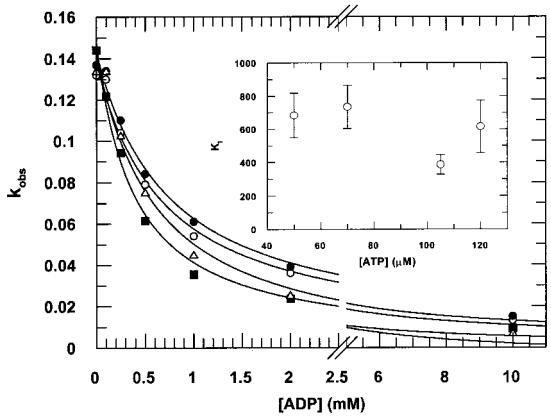

Figure 6. ADP inhibits ATP hydrolysis in single turnover conditions. The rates obtained from fitting the data presented in Figure 4 are shown here. ADP completely inhibits ATP turnover to give an inhibition constant (K_i) of 0.709 (±0.176) mM when fit to a hyperbolic inhibition curve. To probe the mode of inhibition (whether competitive or non-competitive) the concentration of ATP was varied; the inset shows that the K_i is not affected by concentration of ATP, indicating non-competitive inhibition.

hydrolysis (Todd *et al.*, 1994). This observation, coupled with the fact that GroES binding to a GroEL ring is extremely rapid (Burston *et al.*, 1995), would remove this potential short-circuit by preventing nucleotide equilibration and committing bound ATP to hydrolysis. It would also mean that the product complexes (SDL and LDS) would be stable (Jackson *et al.*, 1993; Todd *et al.*, 1994) until the next round of the ATPase cycle when GroES is forced to dissociate (Burston *et al.*, 1995).

Inhibition of the ATPase by ADP in the presence of GroES

It is clear from the above discussion that GroES has profound effects on the association and dissociation of nucleotides, hence it is important to determine the kinetic influence of ADP in reactions where the co-protein is present. The system was examined by mixing a solution of GroES and pyrene-labelled GroEL with ATP in the stopped-flow apparatus (GroEL and GroES do not interact in the absence of adenine nucleotides). The results (Figure 7) are quite different from those in the absence of the co-protein. There is a similar rapid increase in fluorescence owing to the association of ATP and GroES (to form STL), but only a small amplitude decay upon hydrolysis because the product of the reaction is the stable, high fluorescence SDL complex mentioned above (Jackson *et al.*, 1993). The addition of ADP does not inhibit binding of ATP and GroES but it results in a complete abolition of the downward-going reaction transient. Thus, with GroES in the reaction, the fluorescence of pyrenyl:GroEL cannot be used to report ATP hydrolysis. Instead, the hydrolytic rates were measured by the evolution of radio-labelled orthophosphate from the ATP substrate.

Shown in Figure 8 are the observed rates of ATP hydrolysis for GroEL in the absence and presence

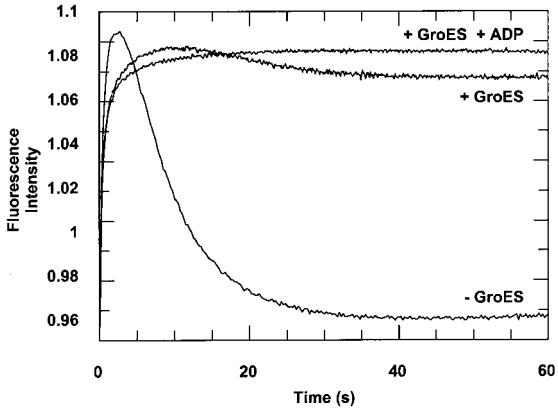

Figure 7. The GroEL:ADP:GroES complex fluorescence masks hydrolysis. The 230 μM GroEL subunits were mixed with a single turnover of ATP (bottom trace), plus a 1:1 ratio of GroES to GroEL complex, with and without 100 mM ADP (top and middle traces, respectively). This highlights the absence of an appreciable hydrolysis signal in the presence of GroES due to the formation of a hyper-fluorescent GroEL:ADP:GroES complex, and reduction of this signal in the presence of ADP.

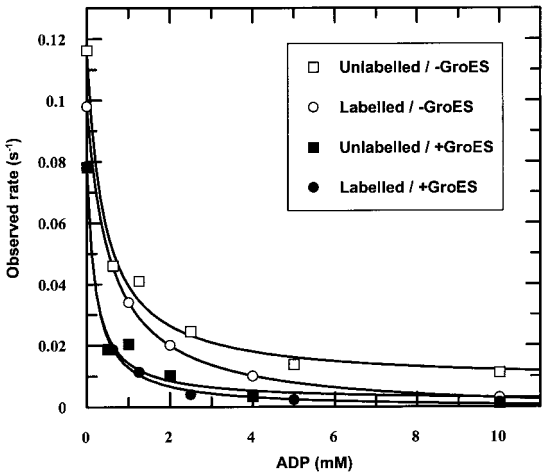

Figure 8. Hydrolytic assays show GroES increases the affinity of the inhibitory ADP. Single turnovers of GroEL (both labelled (circles) and unlabelled (squares)) were performed in the standard buffer as for Figure 3(b) and repeated in the presence of GroES (filled icons) to show the K_i decreases from 0.57 (±0.044) mM to 0.194 (±0.0744) mM (pyrenyl:GroEL) and from 0.41 mM (±0.095) mM to 0.203 (±0.0164) mM (unlabelled GroEL). The experiment was performed with both labelled and unlabelled GroEL to show that labelling has no effect on this aspect of the protein.

of GroES with varied amounts of ADP. Under these conditions, GroES tightens the inhibition constant from approximately 500 to 200 μM and, as in the absence of co-protein, at an infinite concentration of ADP the rate of turnover is zero. Also, as shown by the fact that ADP does not prevent loading of ATP and GroES, inhibition by ADP remains strictly non-competitive. Further, mixing-order experiments show that ADP acts in the same way whether added to a pre-formed GroEL:ATP:GroES complex or whether ADP and ATP are added to a mixture of the two proteins. This confirms that the mode of inhibition in the presence of GroES is the same as that for GroEL alone. Whether or not the addition of GroES commits bound ATP to hydrolysis (thereby avoiding nucleotide equilibration) needs to be addressed.

To test the hypothesis the most direct approach would be to use a method of very rapidly removing any free ATP in the system; such a quench promotes the dissociation and removal of bound but unhydrolysed ATP. Here, we used hexokinase and glucose to remove any unbound ATP rapidly. Rapidly mixed GroEL and ATP, after a given delay time, are subjected to a second rapid mixing agent. In the second mix a large excess of hexokinase and glucose is introduced, hence any ATP released from GroEL will be removed. This would have the effect of promoting dissociation of uncommitted ATP by dragging the equilibrium back towards the free state. In turn, this would be seen as a reduction in pyrene fluorescence, because the removal of ligand promotes a conformational relaxation in GroEL back to the less fluorescent apo state. If, however, ATP becomes fully committed to hydrolysis in the presence of GroES, then the presence of hexokinase should have no effect on the rate of the observed hydrolytic transient.

Shown in Figure 9 are the sequential-mix, stopped-flow fluorescence transients obtained upon mixing GroEL with a single turnover of ATP, followed by quenching with hexokinase and glucose after a given time. These are contrasted with the observed fluorescence transient in the absence of any hexokinase quench (data identical to the heavy line in Figure 3(a)). The addition of a hexokinase quench to the system results in a loss of fluorescence at a rate of approximately 0.44 s^{-1}. Using half the amount of hexokinase this rate is unchanged, showing that hexokinase activity is not limiting (data not shown). Since the rate of hydrolysis is about four times slower than this, it can be concluded that all the ATP bound to GroEL is not committed to hydrolysis.

The rate of 0.44 s^{-1} represents the sum to the rate of hydrolysis (0.11 s^{-1}) and the relaxation (0.33 s^{-1}) from the ''open'' state (or R conformation) to the ''closed'' state (T). In the opposite direction (T-R), the ligand-induced conformational change occurs at 180 s^{-1}. Hence, in the system:

$$\text{GroEL(T)} + \text{ATP} \leftrightarrow \text{GroEL(T):ATP} \leftrightarrow \text{GroEL(R):ATP}$$

The second step is highly favourable ($K_{eq,} = 550$) as

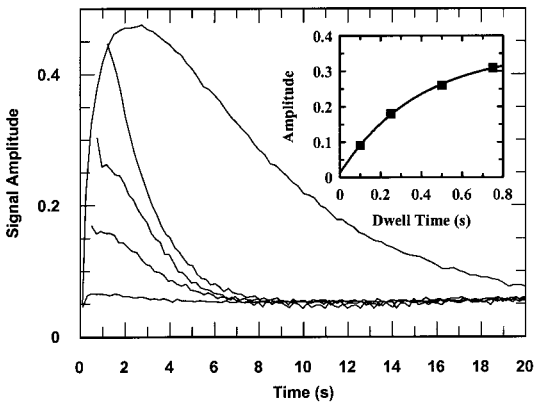

Figure 9. ATP is not committed to hydrolysis with GroEL alone. GroEL was rapidly mixed with a single turnover of ATP and after a given time further mixed by sequential stopped-flow with hexokinase to give final concentrations of 90 μM ATP, 100 μM pyrenyl:GroEL and 2 mg/ml hexokinase. The main graph is an overlay of five transients, the top trace was not mixed with hexokinase but the four below were mixed with increasing dwell time (time before mixing with hexokinase). The transients were fitted to single exponential decays and the amplitudes plotted as a function of dwell time (inset graph). The inset graph was fitted to a single exponential rise to give a rate 2.6 (±0.37) s^{-1}, this is approximately the same as that of the conformational rearrangement in Figure 5.

described (Jackson *et al.*, 1993; Yifrach & Horovitz, 1995).

To show that hexokinase immediately hydrolyses free ATP, the quench was added after variable dwell times. With increasing dwell times the amplitude of the fluorescence transient increases and when plotted against dwell time (Figure 9, inset) the loading of GroEL with ATP is described. The same rate constant for loading is measured as in the experiment of Figure 5. This confirms the validity of using hexokinase as a quenching agent; if the quench were too slow the amplitudes of the transients would not correspond to the fluorescence increase owing to loading of GroEL in the absence of hexokinase (Figure 9, top trace).

To test the hypothesis that GroES commits to ATP to turnover the experiment of Figure 9 was repeated in the presence of GroES equimolar to GroEL. Figure 10 shows three transients, the top and bottom trace define a window of fluorescence and the middle transient describes the effect of hexokinase quench on the GroEL*:ATP:GroES complex (GroEL* refers to GroEL in the R state). In the presence of GroES the observed transient is four times slower (with a value of approximately 0.11 s^{-1}), showing that the release of ATP from the complex is retarded. Therefore, the presence of GroES commits more ATP to hydrolysis.

One possible flaw with the hexokinase quench is that ADP produced by hexokinase could re-associate with GroEL. This was investigated by using an alternative quenching system, alkaline phosphatase. In this case all ATP is hydrolysed to adenosine and phosphate. Figure 11 shows the transients obtained in the presence and absence of GroES; the rates are very similar to those in the presence of hexokinase thereby confirming ATP is more committed to hydrolysis when GroES resides on the complex.

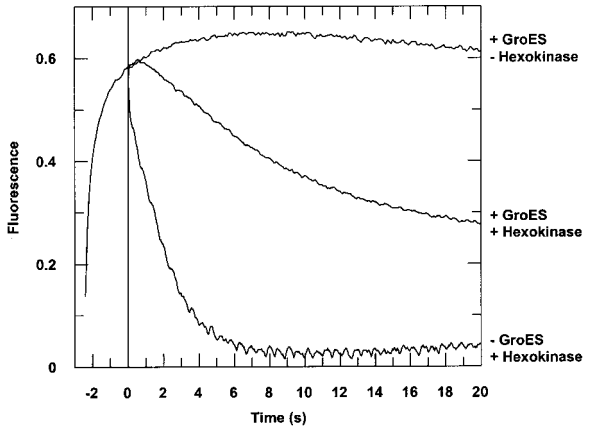

Figure 10. GroES commits ATP to hydrolysis. The experiment described in Figure 9 was repeated in the presence of 1:1 GroES to pyrenyl:GroEL complex. The bottom trace shows the effect of hexokinase (mixed after 2.5 seconds) without GroES (identical to Figure 9) and the top trace is a stopped-flow transient in the presence of GroES but no hexokinase (as in Figure 7). These transients define a window of 0 and 100% ADP fluorescent states, respectively. In the presence of both GroES and hexokinase the middle trace was fitted to a single exponential to give a rate of decay of 0.112 (±0.001) s^{-1}.

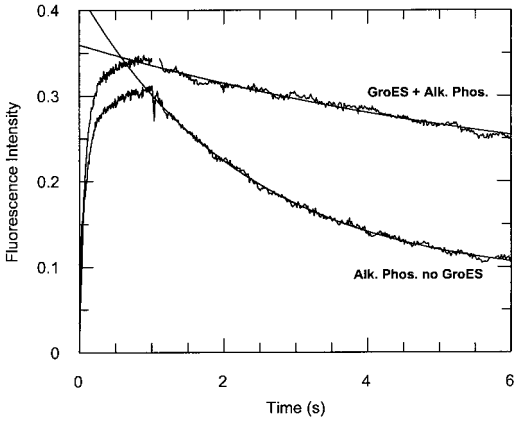

Figure 11. Alkaline phosphatase shows ADP produced by hexokinase does not affect quenching efficiency. The 20 μM subunits of pyrenyl-GroEL with or without 3.6 μM GroES (complex) in the standard buffer were mixed with an equal volume of 1 mM ATP by sequential stopped-flow. A one-second dwell time was allowed for nucleotide binding and the subsequent fluorescence increase before a further mix with 90 units of alkaline phosphatase (final concentrations are 2.75 μM GroEL and 275 μM ATP). The fitted lines correspond to rates of 0.436 (±0.006) s^{-1} and 0.137 (±0.002) s^{-1} in the absence and presence of GroES, respectively.

The preferred reaction pathway

If the reaction scheme shown in Figure 1 is edited according to the constraints induced by commitment to and inhibition of hydrolysis, the system simplifies to that shown in Figure 12(a). Beginning arbitrarily at DLT, the rapid and preferential association of GroES with the ATP binding ring (Burston *et al.*, 1995; Hayer-Hartl *et al.*, 1995) leads to the formation of DLTS. This complex is shown here to be hydrolytically inactive and therefore halts the progress of the cycles. In order for the cycle to proceed, ADP must dissociate to form the hydrolytically active LTS. In the presence of high ratios of ATP:ADP, TLTS is likely to form, this complex is also hydrolytically active but has ATP bound to both rings. This raises the possibility of turnover on either ring, if turnover occurs in *trans* then DLTS forms and hydrolysis will proceed on one ring. This has been shown not to be the case, since GroES (Burston *et al.*, 1995) and polypeptide (Weissman *et al.*, 1994; Ranson *et al.*, 1997) are released once per cycle. In addition to this, by examining the behaviour of a mixed-ring, mutant complex with one ring unable to cleave ATP at a significant rate and the other unable to bind GroES (Rye *et al.*, 1997), it was shown that hydrolysis in *cis* precedes that in *trans*. As a consequence the

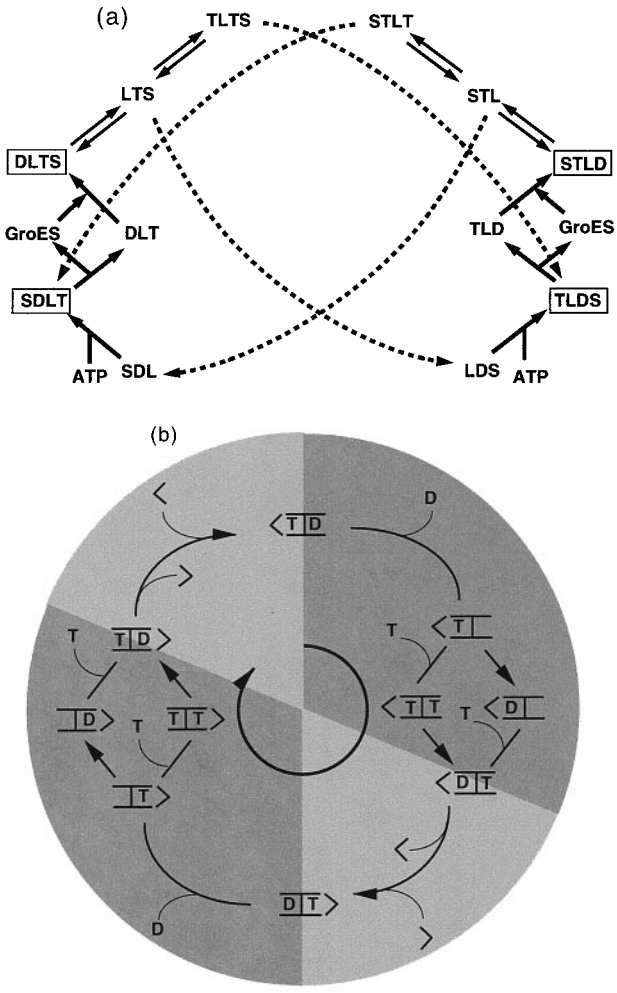

Figure 12. Summary of the likely GroE hydrolytic cycle. (a) This scheme is set out as in Figure 1 but all steps that are disallowed by ADP inhibition and commitment to hydrolysis have been removed. From the data of Rye *et al.* (1997) we infer that the presence of ATP in the SDLT and TLDS complexes promotes GroES dissociation from the opposing ring. The hydrolytically inactive STLD and DLTS complexes are shown in boxes. (b) Here the system of reactions is represented as an orthodox cycle with the GroES-switching phases shown on a light grey background and the nucleotide-switching phases shown on mid grey.

TLTS complex preferentially hydrolyses to form TLDS.

In the case where ATP does not occupy the *trans* ring, the LTS complex will yield LDS which, in turn, can bind ATP in *trans* to give TLDS. Hence, in either case nucleotide is switched between rings resulting in the following partial reaction:

$$\text{DLTS} \rightarrow \text{TLDS}$$

The formation of TLDS leads to a further hydrolytic dead end owing to non-competitive inhibition by ADP. However, the presence of ATP in *trans* stimulates the dissociation of GroES from the TLDS complex (Rye *et al.*, 1997). Hence, TLDS must first be converted to TLD which can then rapidly accept GroES on the opposite ring to yield STLD. In this partial reaction, the fact that ATP hydrolysis is blocked leads to a pause during which the co-protein is redistributed from one ring to the other. The formation of STLD is the symmetric equivalent of DLTS signifying the completion of a hemi-cycle in which the nucleotide and then GroES are switched between rings.

In summary, a combination of (a) ATP-driven release of GroES, (b) non-competitive inhibition by ADP, and (c) the increase in commitment of *cis* ATP to hydrolysis, provides both directionality and an alternating function of each ring; a process vital to GroEL function (Weissman *et al.*, 1995, 1996; Mayhew *et al.*, 1996).

Although we do not address this issue with the experiments reported here, examination of the preferred reaction sequence illustrated in Figure 12(a) prompts the question of coupling the ATPase cycle to the binding and release of unfolded protein substrates. The kinetic scheme in Figure 12(a) can be simplified to that shown in Figure 12(b) to provide an insight into the properties of complexes in the steady-state. Several important factors help to define these species.

(1) To define the protein binding species it is necessary to ascertain the degree of interaction of non-native protein with GroEL. An unliganded ring (with respect to nucleotide) has been shown to have the highest affinity for non-native protein followed by an ADP liganded ring and then the ATP conformation (Staniforth *et al.*, 1994; Yifrach & Horovitz, 1996). It has further been shown that negative co-operativity will tend to maintain one ring in a high protein-affinity state and the other in a low affinity state (Yifrach & Horovitz, 1996). However, the affinity of a ring in *trans* to GroES is ill-defined.

(2) Folding has been shown to occur preferentially in an encapsulated state with ATP in *cis* (i.e. in S(P)TLX, where (P) is the substrate protein and X is unliganded, ADP or ATP). Conversely, TLD(P)S and S(P)DLT do not support folding due to the presence of ADP in *cis* (Burston *et al.*, 1996; Weissman *et al.*, 1996).

(3) At infinite GroEL/GroES concentrations protein folding is rapid (Ranson *et al.*, 1997); this would appear to preclude TLD(P)S and S(P)DLT as the principle steady-state complexes owing to the resultant inhibition of encapsulated folding.

(4) The substrate protein is ejected once per cycle (Weissman *et al.*, 1994; Ranson *et al.*, 1997) and this may occur from a transient or a major steady-state complex.

The candidates for predominant steady-state complexes are the trio of species within the equilibrium system which precede ATP turnover in *cis*, i.e. DLTS, LTS and TLTS and their ''mirror'' images. In addition the SDLT complex that precedes the GroES switching process may also

accumulate. Using the criteria discussed above, it is more likely that the DLTS/LTS/TLTS trio constitute the major species, since these can support encapsulated folding in the ATP-occupied *cis* cavity and are also capable of accepting substrates in the *trans* ring.

It is interesting to note that in conditions of cellular stress the cycle is likely to slow down because of the accumulation of ADP and the resultant inhibition of the ATPase activity. This may provide a method of avoiding unnecessary wastage of ATP and also create a static "buffer" for unfolded proteins which are absorbed onto chaperonin complexes but not ejected until the ATP/ADP ratio is restored.

Materials and Methods

Standard conditions

All experiments described here were performed in a standard reaction buffer containing 50 mM triethanolamine hydrochloride (pH 7.5), 50 mM KCl and 20 mM $MgCl_2$. Unless stated otherwise, all experiments were performed at 25°C.

Proteins and reagents

GroEL and GroES were purified by a modification of the method described by Burston *et al*. (1995). GroEL and GroES were co-expressed in *E. coli* MC1061 cells from the pND5 plasmid. Cells were cultured for 18 hours at 37°C, heat-shocked at 42°C for one hour to enhance expression of the chaperonins, and then harvested after a further one hour at 37°C. The cells were lysed by sonication in 50 mM Tris-HCl (pH 8.5), 5 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF), and the cell debris was removed by centrifugation. DNA was digested in the crude cell extract by adding 10 mM $MgCl_2$ and 0.1 mg ml^{-1} DNase, and leaving for 30 minutes at room temperature. After a further centrifugation step to remove precipitated material, and readjustment to pH 8.5, the supernatant was loaded onto a Q-Sepharose anion-exchange FPLC column equilibrated in 50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 20 mM $MgCl_2$. The column was washed with two column volumes of the same buffer before bound proteins were eluted using a four column volume gradient from 0 mM to 1000 mM NaCl. GroES eluted at 400 mM, while GroEL eluted at 650 mM NaCl. The GroEL-containing fractions were pooled and HPLC grade methanol was added to 25% (v/v) before application to an Sephacryl S-200 HR FPLC gel filtration column pre-equilibrated in 50 mM Tris (pH 8.0), 5 mM EDTA, 20 mM $MgCl_2$ and 25% (v/v) HPLC grade methanol. GroEL-containing fractions were pooled and dialysed against 200 volumes of the standard buffer containing 0.2 mg ml^{-1} acid-washed charcoal, before being stored as a precipitate in 70% saturated ammonium sulphate. Fractions containing GroES were further purified by adding ammonium sulphate to a concentration of 1.5 M and loading onto a Poros PE hydrophobic exchange column (PerSeptive Biosystems). Pure GroES was eluted using a reverse gradient of 1.5 M to 0 M ammonium sulphate, run over 25 column volumes. The pure protein was stored as a precipitate in 70% saturated ammonium sulphate.

ATP and ADP were obtained from Boehringer Mannheim and stored at 4°C. Stock solutions were made up in the standard buffer and the pH readjusted to 7.5 before being stored at −80°C. Final stock nucleotide concentrations were determined spectrophotometrically using a molar extinction coefficient of 15,300 M^{-1} cm^{-1} for adenine at 280 nm. [γ-^{32}P]ATP was obtained from Amersham Life Sciences (UK). Hexokinase (baker's yeast type C300), glucose and *N*-(1-pyrenyl)maleimide were obtained from Sigma (Poole, Dorset, UK). All other buffers and reagents were obtained from BDH and were analytical grade.

Labelling of GroEL with *N*-(1-pyrenyl)maleimide

The sub-stoichiometric labelling of the GroEL oligomer with *N*-(1-pyrenyl)maleimide was performed essentially as described (Jackson *et al*., 1993; Burston *et al*., 1995). Briefly, 10 ml of 350 μM GroEL (monomer concentration) in the standard buffer were rapidly mixed with 78 μl of *N*-(1-pyrenyl)maleimide dissolved in HPLC grade acetonitrile (stock concentration of 3.2 mM). The labelling reaction was then left to go to completion (two hours) in the dark. The resulting pyrenyl:GroEL conjugate was precipitated by addition of ammonium sulphate to 70% saturation. The precipitated protein was then centrifuged at 13,000 rpm for 15 minutes and the resulting pellet was resuspended in the standard buffer, before being dialysed against 3×200 volumes of the standard buffer containing 1 mg ml^{-1} acid-washed charcoal to remove any unreacted fluorescent dye. The concentration of the pyrenyl:GroEL conjugate was assessed using a molar extinction coefficient of 34,000 M^{-1} cm^{-1} of pyrenyl-cysteine (Kouyama & Mihashi, 1981). This confirms a labelling stoichiometry of 0.92 pyrene groups per GroEL oligomer in the pyrenyl:-GroEL conjugate.

Single-mix and sequential-mix stopped-flow measurements

Single and sequential-mix stopped-flow experiments were performed using a thermostatically controlled Applied Photophysics (Leatherhead, UK). Samples were excited using monochromated light at a wavelength of 343 nm, and emitted light was selected using a WG360 filter, cutting off all light with a wavelength below 360 nm. Cysteinyl pyrene has peak absorbance at 343 nm and fluorescence at 375 nm. All reactions were performed at 25°C, and in the standard buffer. Each recorded transient is an average of at least three individual reactions.

Sequential-mix measurements were obtained using the same apparatus reconfigured for sequential-mix stopped-flow, and using the same optical and data collection parameters.

Assay for ATP hydrolysis by GroEL

The assay for the hydrolysis of ATP by GroEL was performed as described (Burston *et al*., 1995). Briefly, the required concentrations of nucleotide (containing ~10 μCi of [γ-^{32}P]ATP) and GroEL were mixed, and aliquots removed at various times over the course of the reaction. Each aliquot was quenched by addition of ten volumes of 40 mM HCl containing 30 mg ml^{-1} acid-washed charcoal at the time of removal. Any unhydrolysed ATP, and ADP bind to the charcoal leaving hydrolysed $^{32}P_i$

groups in solution. After removal of charcoal (and therefore of bound nucleotide) by centrifugation, the amount of $^{32}P_i$ in the supernatant was assayed by Čerenkov radiation counting.

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