Hydrolysable ATP is a requirement for the correct interaction of molecular chaperonins cpn60 and cpn10

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Over recent years the binding ability of the molecular chaperone cpn60 ($GroEL_{14}$) and its co-chaperone cpn10 ($GroES_7$) has been reported to occur under an assortment of specific conditions from the use of non-hydrolysable ATP analogues (namely adenosine 5′-[γ-thio]triphosphate) to requiring hydrolysable ATP for any interaction to occur. We have investigated this further using the molecular hydrodynamic methods (hydrodynamic bead modelling, sedimentation-velocity analytical ultracentrifugation and dynamic light-scattering), allowing the process to be followed under physiologically relevant dilute solution conditions, combined with absorption spectrophotometry to determine $GroES_7$–$GroEL_{14}$ interaction through the rate inhibition of the cpn60's ATPase activity by $GroES_7$. The results found here indicate that the presence of hydrolysable ATP is required to facilitate correct $GroES_7$ interaction with $GroEL_{14}$ in solution.

Key words: ATP[S], analytical ultracentrifugation, dynamic light-scattering, hydrodynamic bead modelling.

INTRODUCTION

The *Escherichia coli* molecular chaperone $GroEL_{14}$ and its co-chaperone $GroES_7$ belong to a ubiquitous class of proteins known as chaperonins. They are also known as hsp60 (heat-shock protein 60) and hsp10 on account of their ability to protect proteins against denaturation under conditions of abnormally high cellular temperature. The functionality of these proteins is attributed to their role in the folding of newly synthesized and stress-denatured proteins into their correct conformation [1] and they are homologous to the cpn60 and cpn10 proteins of eukaryotic mitochondria and chloroplasts [2]. $GroEL_{14}$ is an oligomeric large tetradecamer composed of two stacked heptameric rings that are made up of identical subunits of about 58 kDa [3–5] arranged with seven-fold cylindrical symmetry [6]. $GroEL_{14}$ has been demonstrated to assist in the refolding of a wide variety of proteins and is able to interact with over half of the proteins from *E. coli* [7]. The ability of $GroEL_{14}$ to bind and fold nascent proteins has been shown to be, in most cases, dependent on its interaction with the co-chaperonin $GroES_7$ [8]. $GroES_7$ is a seven-membered ring composed of identical 10 kDa subunits, which, in the presence of Mg^{2+} and ATP, can form stable complexes by capping the apical regions of the $GroEL_{14}$ molecule.

It has been known for some time that $GroEL_{14}$ possesses K^+-dependent ATP-ase activity [9], and several studies have shown that the apical domains of the $GroEL_{14}$ swing outwards upon ATP and $GroES_7$ binding [3,10], indicating that the exposure of the hydrophobic substrate-binding domains is triggered by ligand-induced conformational changes [2]. However, conflicting published data suggest a range of nucleotide requirements for $GroES_7$ to successfully interact with $GroEL_{14}$: from the use of non-hydrolysable ATP analogues [11–13] to requiring hydrolysable ATP for binding and folding to proceed [9,14]. For this reason in the present study we have investigated the complex formation of $GroEL_{14}$ and $GroES_7$ in solution in the presence of both ATP and the non-hydrolysable ATP analogue 5′-[γ-thio] triphosphate (ATP[S]), using a combination of sedimentation-velocity analytical ultracentrifugation and dynamic light-scattering (also known as photon correlation spectroscopy).

EXPERIMENTAL

Sample preparation

The preparation of the $GroEL_{14}$ was as described by Gibbons et al. [2]. The preparation was stored as a precipitate in a 75%-satd. $(NH_4)_2SO_4$ buffer containing 50 mM triethanolamine, pH 7.5, 50 mM KCl, and 20 mM MgCl. The $GroES_7$ sample was prepared as described in [13] and supplied as a solution in Tris/HCl (pH 7.7)/100 mM NaCl.

Prior to use, small quantities of the precipitated $GroEL_{14}$ protein solution were centrifuged at 13600 ***g*** (base of cell) for 15 min using a microcentrifuge. The supernatant was then removed and discarded. Solutions of both proteins were dialysed overnight at 4 °C against 1 litre of Tris/HCl buffer, pH 7.7, containing 100 mM Tris/HCl, 50 mM KCl and 20 mM $MgCl_2$ (BDH Laboratory Supplies, Poole, Dorset, U.K.). Samples were then adjusted to give molar ratios of $GroES_7$ and $GroEL_{14}$ of 2:1 respectively using the same buffer with addition of ATP or ATP[S] at either 2, 1 or 0.5 mM concentrations. All hydrolysable ATP studies were carried out using a linked-enzyme ATP-regenerating system as described below and in [5,15,16].

The concentration of the dialysed sample was determined by absorption spectroscopy at 280 nm, using the Beer–Lambert

Abbreviations used: cpn60, chaperone 60 ($GroEL_{14}$); cpn10, cpn60's co-chaperone ($GroES_7$); ATP[S], 5′-[γ-thio]triphosphate; PK, pyruvate kinase; PEP, phosphoenoylpyruvate; LDH, lactate dehydrogenase.

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Table 1 Estimated properties of $GroEL_{14}$ and $GroEL_{14}$–$GroES_7$ complexes based on the amino acid sequence at 20 °C in 100 mM Tris/HCl buffer, pH 7.7

Molecule	M_r	$\bar{v}$ (ml/g)	Estimated hydration (g/g)	Charge (Z) at pH 7.0	pI	ϵ_{280} (ml · g^{-1} · cm^{-1})
$GroEL_{14}$	800 000	0.7420	0.4303	−260	5.102	183
$GroEL_{14}$–$(GroES_7)_1$	874 000	0.7424	0.4339	−288	5.099	259
$GroEL_{14}$–$(GroES_7)_2$	950 500	0.7430	0.4364	−315	5.097	322

equation and an absorption coefficient of 183 ml · g^{-1} · cm^{-1} used for $GroEL_{14}$ and 143 ml · g^{-1} · cm^{-1} used for $GroES_7$ as calculated from the amino acid compositions (Table 1).

Predicted properties from amino acid composition

Some molecular properties based on the amino acid composition were calculated prior to analysis of the ultracentrifuge data. For this the routine SEDNTERP (where the user can either enter the amino acid composition data manually or direct from a file) was used, based on [17]. This routine allows an estimate of the monomer molecular mass, the partial specific volume, the pI, the charge (valency), Z, at a specified pH, and the absorption coefficient, ϵ, at 280 nm (Table 1).

Sedimentation-velocity analysis

All sedimentation-velocity experiments were performed in the XL-I ultracentrifuge using interference optics (Beckman Scientific Inc, Palo Alto, CA, U.S.A.). Double-sector cells of 12 mm optical pathlength were used with 390 μl of solution in one sector and 400 μl of solvent after dialysis to equilibrium in the other. The loaded ultracentrifuge cells were then placed in a four-hole titanium rotor and run at a rotor speed of 128 800 $\boldsymbol{g}$ (40 000 rev./min). The temperature was set at 20 °C throughout. Sedimentation coefficients were determined from the $g(s^*)$ profile using time-derivative software (DCDT+) developed by Dr J. Philo (Biotechnology and Software Consulting, Thousand Oaks, CA, U.S.A.) ([18] following [19]) and were corrected to standard conditions ($s_{20,w}$) of water as the solvent and a temperature of 20 °C according to the relationship [20]:

$$s_{20,w} = s_{T,b}\left(\frac{\eta_{T,b}}{\eta_{20,w}}\right)\left(\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho_{T,b}}\right) \quad (1)$$

where $s_{T,b}$ was taken as being equal to the maximum in the $g(s^*)$ profile [$g(s^*)_{max}$] and η, $\bar{v}$ and ρ are the viscosity, the partial specific volume and the density of the solvent respectively. T and b refer to the temperature and buffer used for the particular experiments. The $g(s^*)$ profile means the Gaussian distribution of sedimentation coefficients, and the asterisk denotes that this distribution is uncorrected for translational diffusion.

Theoretical bead-model analysis to determine the solution conformation of the $GroEL_{14}$–$GroES_7$ complex formation

Using bead-modelling-analysis software (SOLPRO [21]), where each subunit is represented as a sphere from its Cartesian co-ordinates, we can evaluate the principal hydrodynamic properties of the various proposed oligomeric species in solution. The calculation involves various stages: calculation of the 'Perrin' function or 'frictional ratio due to shape', P (P is directly related to the shape, via, for example, the axial ratio of the equivalent ellipsoid), followed by an evaluation of the translational frictional ratio, f/f_0 (ratio of the frictional coefficient of the molecule to that of a sphere of the same mass and anhydrous volume) using the following method:

Table 2 Theoretical values of P, f/f_0 and $s^0_{20,w}$ for asymmetrical and symmetrical $GroEL_{14}$–$GroES_7$ structures

Complex	P	f/f_0*	M_r	$s_{20,w}$ (S)*	(s_n/s_{EL}) ratio
$GroEL_{14}$	1.175	1.368	805 000	21.50	1.00
$GroEL_{14}$–$(GroES_7)_1$	1.164	1.357	874 000	23.06	1.07
$GroEL_{14}$–$(GroES_7)_1$	1.153	1.345	950 500	24.55	1.14

* Based on $\delta = 0.4303$, 0.4339, 0.4364 g/g and of 0.7420, 0.7424 and 0.7430 ml/g for $GroEL_{14}$, $GroEL_{14}$–$(GroES_7)_1$ and $GroEL_{14}$–$(GroES_7)_2$ respectively.

$$f/f_0 = P\left[\frac{\bar{v}+\left(\dfrac{\delta}{\rho_0}\right)}{\bar{v}}\right]^{1/3} \quad (2)$$

where δ is hydration, $\bar{v}$ is the partial specific volume and ρ_0 is the density of water as the solvent. The sedimentation coefficients for any given subunit formation can then be determined from the derived translation frictional coefficient as follows:

$$s_{20,w} = \left[\frac{\left(\dfrac{4\pi N_A}{3\bar{v}M}\right)^{1/3}}{\left(\dfrac{f}{f_0}\right)}\right]\left[\frac{M(1-\bar{v}\rho_0)}{N_A 6\pi\eta_0}\right] \quad (3)$$

where N_A is Avagadro's number, M is molecular mass and η_o is the viscosity of water as the solvent. In Table 2 we have calculated from SOLPRO the theoretical values of P, f/f_0, $s_{20,w}$ and the sedimentation-coefficient ratios (s_n/s_{EL}) for a $GroEL_{14}$, $GroEL_{14}$–$(GroES_7)_1$ and a $GroEL_{14}$–$(GroES_7)_2$ complex (Figure 1).

GroEL ATPase activity

The ATPase activity of $GroEL_{14}$ was carried out using a linked-enzyme ATP-regenerating system [pyruvate kinase (PK), phosphoenoylpyruvate (PEP) and lactate dehydrogenase (LDH) (Sigma–Aldrich Co. Ltd. Poole, Dorset, U.K.) as described by Emes and Rowe [22]. Several studies have been carried out using this system, showing it to be non-interactive with the GroE machinery ([5,15,16]. Sample dilutions were made directly into a reaction buffer consisting of 100 mM Tris/HCl, 50 mM KCl, 20 mM $MgCl_2$, 8 mM PEP, 2.5 mM NADH and 75 μl of premixed enzyme solution containing 750 units/ml PK and 1000 units/ml LDH, to give final concentrations of 1 μM $GroEL_{14}$ and 2 μM $GroES_7$. The samples were loaded into quartz cells and, once a steady baseline was achieved, ATP at 0.5 mM was added and the conversion of ATP into ADP was then monitored as the loss of A_{340} due to the oxidation of NADH to NAD^+.

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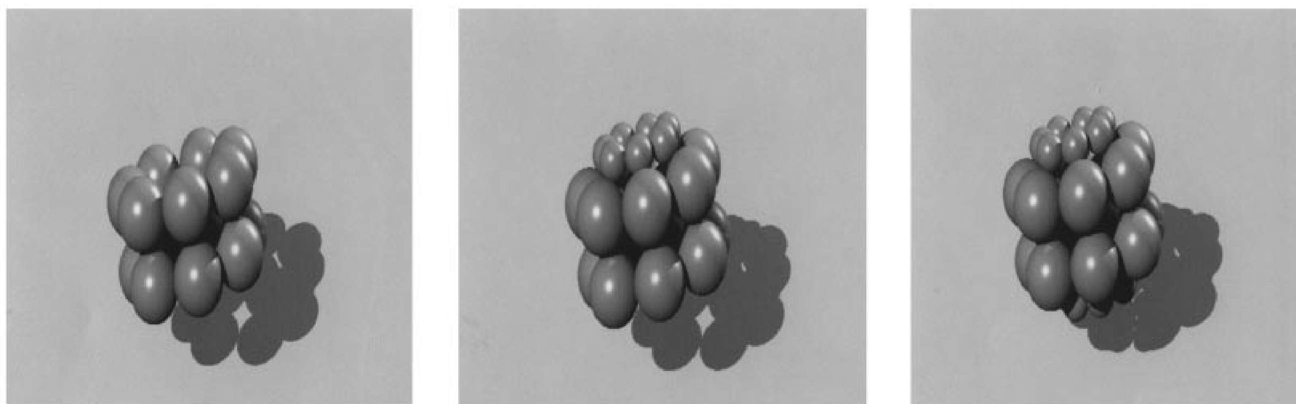

Figure 1 Bead-model representation of the $GroEL_{14}$, $GroEL_{14}$–$(GroES_7)_1$ and $GroEL_{14}$–$(GroES_7)_2$ complexes

The model was produced using a public domain shareware rendering package (Polyray v1.8), provided by Alexander Enzmann (http://www.povray.org).

Photon correlation spectroscopy

Dynamic light-scattering measurements were carried out in triplicate for each sample using a DP801 TC 90°-fixed angle molecular-sizing instrument (Protein Solutions, High Wycombe, Bucks., U.K.). All samples and buffers were as described above and filtered prior to use. A 100 μl portion of each sample was injected. An ATP-regenerating system was used as described above. Diffusion coefficients (D) and the hydrated radius (R_h) were determined using online software provided by the manufacturers for this purpose (Dynals, v4.0).

RESULTS

GroEL ATPase activity

In this investigation, the PK/LDH linked enzyme assay was employed [22]. The ATPase activity of $GroEL_{14}$ provides rate-limiting amounts of ADP, which allows the ATPase activity to be determined by spectrophotometry following the biochemically coupled loss of absorbance of NADH at 340 nm as it is oxidized to NAD^+ by lactate dehydrogenase [22]. The activity of the system was established and calibrated using aliquots of standard ADP as a control, which gave an immediate loss of absorption with no $GroEL_{14}$ present, indicating good enzymic activity of the regenerating system.

The absorbance of 100 μM NADH at 340 nm was estimated to be around 0.622 on the basis of a molar absorption coefficient of 6220 litre · mol^{-1} · cm^{-1} (i.e. $A_{340} = 0.0001 \times 6220 = 0.622$). Taking baseline levels into account, the true values of the samples used here containing 100 μM was given to be 0.63 for the $GroEL_{14}$ sample and 0.6 for the $GroEL_{14}$–$GroES_7$ sample showing consistency in NADH concentration used.

For a 1 ml sample of $GroEL_{14}$ at 0.5 μM, linear activity was shown throughout the experiment, with a rate of NADH oxidation (ΔA units/min) of 0.12/min, with the full 100 μM NADH being oxidized in $\approx$ 5 min. This equates to 19.3 μmol of NADH oxidized/min, i.e. 0.12/6220 = 19.3 μmol/min.

The concentration of a 1 ml $GroEL_{14}$ sample was 0.5 μM or 0.4 mg/ml. Assuming an equimolar amount of ATP is hydrolysed per NADH oxidation, we can conclude that the specific ATPase activity of $GroEL_{14}$ in units (the amount of enzyme required to convert 1 μmol of substrate/min) [23] is equivalent to the amount of NADH oxidized/min, i.e. 19.3/0.4 mg or 48.25 units/mg of GroEL.

The rate found for 0.5 μM (0.4 mg/ml) $GroEL_{14}$ in the presence of 1 μM (0.071 mg/ml) $GroES_7$ was again linear throughout the assay, but markedly inhibited, with NADH oxidation being reduced to 8.04 μM/min. This gives a reduced specific ATPase activity of 20.1 units/mg of $GroEL_{14}$ in the presence of 0.18 mg of $GroES_7$. This reduction in ATPase activity is 2.4 times lower than $GroEL_{14}$ on its own or represents about a 58.5 % decrease in activity.

Photon correlation spectroscopy

Photon correlation spectroscopy, which operates by measuring the temporal fluctuations in the light scattered by the particles to determine the level of correlation of the time-varying signal [24], was employed to measure any change in relative size of the $GroEL_{14}$, depicted by the hydrated radius (R_h), during $GroEL_{14}$ and $GroES_7$ interaction. The analysis of $GroEL_{14}$ and $GroES_7$ individually gave values for R_h of 8.54 ± 0.2 nm and 3.91 ± 0.1 nm respectively as baseline values. Measurements were also taken with the ATP-regenerating system present, which gave no significant change in radial size. It was therefore assumed no interaction with the enzyme system used here was taking place.

As Figure 2 shows, virtually no change in R_h of the principle scattering component was detected with the $GroEL_{14}$ and $GroES_7$ sample ($R_h = 8.49 \pm 0.18$ nm) or the $GroEL_{14}$–$GroES_7$ incubated with the non-hydrolysable ATP[S] ($R_h = 8.48 \pm 0.058$ nm) when compared with the standard $GroEL_{14}$-only sample ($R_h = 8.54 \pm 0.2$ nm). This indicates that no interaction was taking place between the $GroEL_{14}$ and $GroES_7$ molecules, whether in the absence of nucleotides or in the presence of the non-hydrolysable ATP analogue. However, with the addition of ATP, a significant increase in R_h is shown, with an 8 % increase occurring with 0.5 mM ATP and a 16 % increase occurring with the inclusion of 2 mM ATP. Assuming a spherical model, this corresponds to an increase in volume of $\approx$ 22 % and $\approx$ 41 % respectively. This is in very good agreement with results found from X-ray-crystallographic data [6,25], which, using the formula $v = \int_b^a \pi[f(y)]^2 \, dx$, gives $\approx$ 24 and $\approx$ 48 % (assuming symmetry) increase for $GroEL_{14}$ with one $GroES_7$ and two $GroES_7$ molelcules bound

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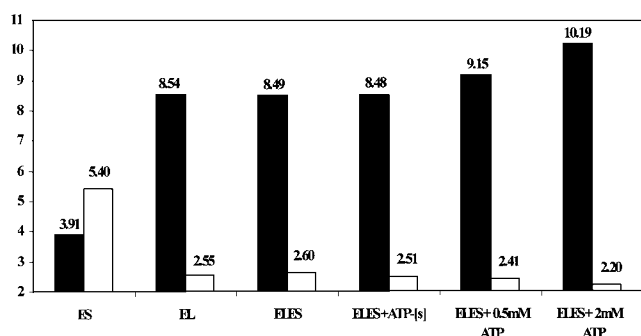

Figure 2 R_h **(nm) (black bars) and** $D_{20,w}$ **($\times 10^{-7}$) (white bars) values for GroEL and GroES with ATP determined using dynamic light-scattering**

respectively. This suggests that, in the presence of relatively low concentrations of ATP (0.5 mM), the GroEL–GroES complex shows mainly asymmetric 1:1 $GroEL_{14}$–$(GroES_7)_1$ stoichiometry, whereas at higher ATP concentrations (2 mM) the complex is shifted to give mainly symmetrical 2:1 $GroEL_{14}$–$(GroES_7)_2$ stoichiometry (Figure 1).

Sedimentation velocity

Time-derivative analysis of sedimentation-velocity data determined for the $GroES_7$ and $GroEL_{14}$ molecules separately gave $s^0_{20,w}$ values of ≈ 4 S and 21.5 S respectively, which corresponds well with the findings of Behlke and co-workers [13] (Figures 3a and 3b). A 2:1 molar ratio of $GroES_7$ and $GroEL_{14}$ respectively gave two main peaks (Figure 4a) at 4.01 S and 21.87 S, showing no indication of a complex interaction between the two molecules. Similarly, the addition of 0.5 mM or 1 mM ATP[S] (Figure 4b) also shows two sharp peaks of 3.8 S and 22.3 S, corresponding to the unbound $GroES_7$ and $GroEL_{14}$ molecules. The addition of 2 mM ATP gave a slightly different picture (Figure 4c), and, owing to the ATPase activity of $GroEL_{14}$, which would mean the ATP was hydrolysed completely to ADP before the sedimentation analysis was started, we consider these results to be in the presence of ADP. As Figure 4(c) shows, there is again a single peak at ≈ 4 S corresponding to unbound $GroES_7$, with a second broader peak that no longer fits to a single species. This peak is a good fit to a two-species model of 21.6 S and 23.5 S giving an s_n/s_{EL} ratio of 1.08, which corresponds to within 2% the theoretical value determined from bead modelling (Table 2) of 1.07 for the asymmetrical 1:1 $GroEL_{14}$–$(GroES_7)_1$ complex. In addition, a forced molecular-mass analysis, where the expected molecular masses for this sample were fixed, of the same data echoes back both similar sedimentation-coefficient values and a reasonable fit (results not shown), leading to a degree of confidence in this evaluation. A repeat evaluation of $GroEL_{14}$, this time with an ATP-regenerating system included (Figure 5a), revealed no change in the sharp peak given at 22.3 S, and gives two separate smaller peaks representing the enzyme system. This indicates that no interaction between the enzyme system and $GroEL_{14}$ is taking place under the conditions used here. Although (as the peaks for the enzyme system overlap those for the $GroES_7$ molecule) evaluation of $GroES_7$ in the presence of the enzyme is not possible, this does not, however, distract from the evaluation of the peak at the higher s^* value. The addition of the enzyme system to the $GroEL_{14}$–$GroES_7$–ATP sample (Figure 5b) shows that the main peak broadens, which gives a reasonable fit to a two-species model with sedimentation coefficients of 21.15 S and 24.67 S, giving an s_n/s_{EL} ratio of 1.16, which is again within 2% of the ratio of the theoretical value determined from bead modelling (Table 2) for the $GroEL_{14}$–$(GroES_7)_2$ complex of 1.14. In addition, the proposed weight of these two species can be determined using the Svedberg equation (eqn 4):

$$M = \frac{sRT}{D(1-\bar{v}\rho)} \tag{4}$$

where D is the diffusion coefficient obtained from dynamic light-scattering, s is the sedimentation coefficient of the individual species obtained from sedimentation-velocity analysis, R is the gas constant and T is the temperature. This gives molecular-mass (M) values for the 21.15 S and 24.6 S components of 803 and

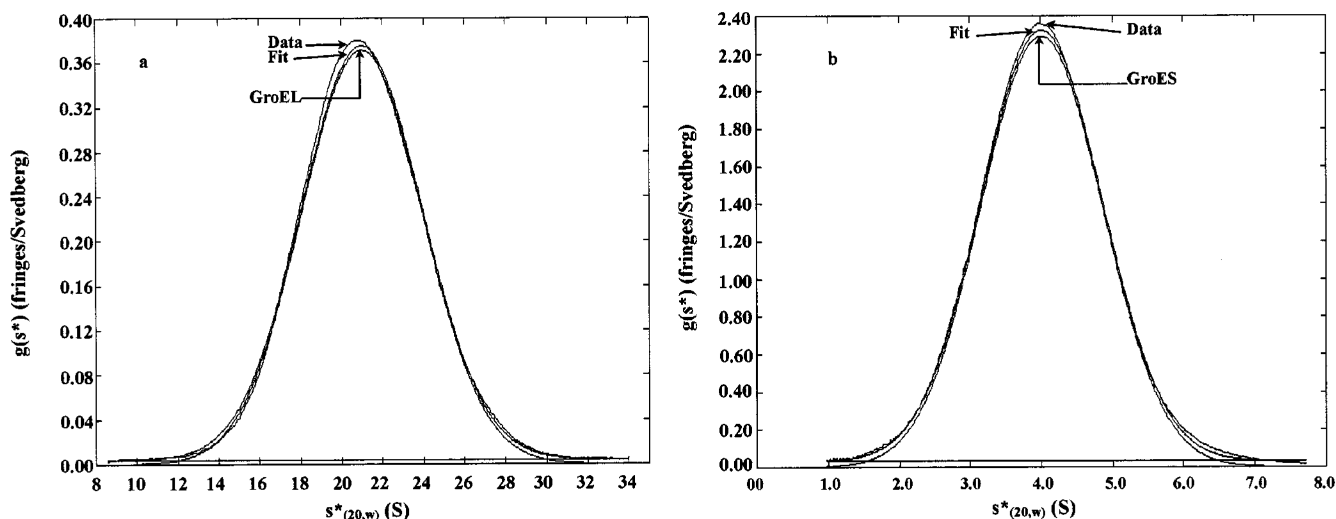

Figure 3 Single-species time-derived fits to (a) $GroEL_{14}$ **($s_{20,w} \approx 21.5$ S) and (b) GroES ($s_{20,w} \approx 4.0$ S)**

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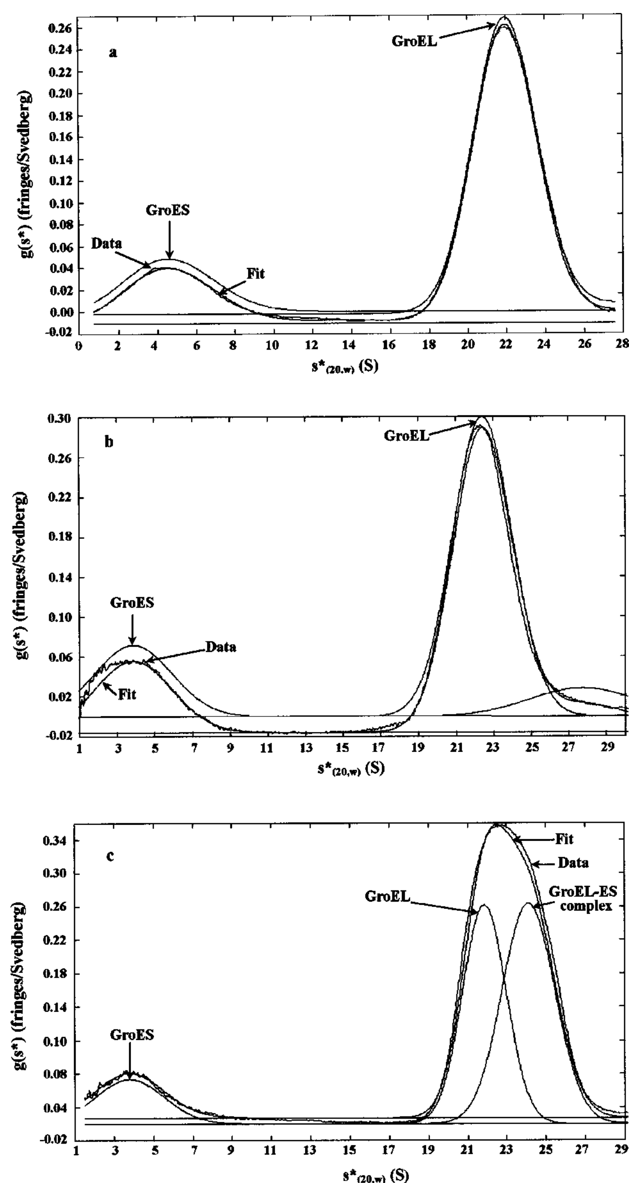

Figure 4 Time-derivative fits to GroEL and GroES complex formations

The Figure shows time-derivative fits to (**a**) 1 : 2 molar ratio of GroEL ($s_{20,w}$ = 21.87 S) and GroES ($s_{20,w}$ = 4.01 S), (**b**) 1 : 2 molar ratio of GroEL ($s_{20,w}$ = 22.30 S) and GroES ($s_{20,w}$ = 3.80 S) with the addition of ATP[S] and (**c**) 1 : 2 molar ratio of GroEL showing complex formation as a two-species fit ($s_{20,w}$ = 21.60 S and 23.50 S) with unbound GroES present ($s_{20,w}$ = 4.00 S) with the addition of ATP.

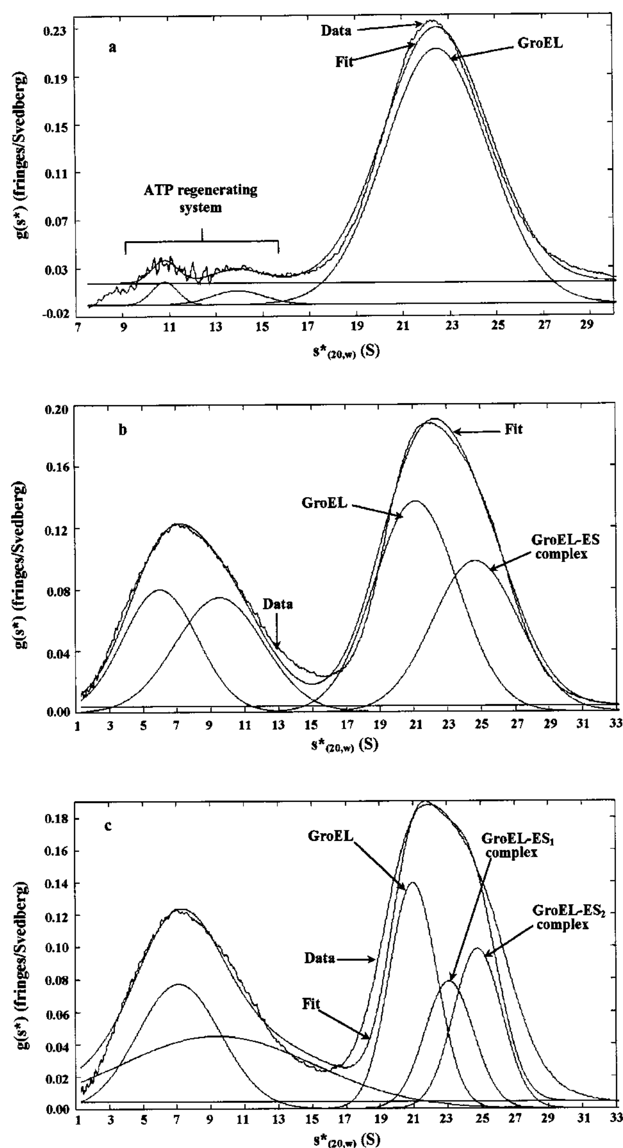

Figure 5 Time-derivative fits to GroEL and GroES complex formations

The Figure shows time-derivative fits to (**a**) GroEL including the PEP–lactate ATP-regenerating system, indicating no interaction between the enzyme system and the GroEL machinery, (**b**) 1 : 2 molar ratio of GroEL and GroES with the addition of ATP, incorporating a two-species free fit of sedimentation coefficients and (**c**) a forced molecular-mass fit to a three-species model of a 1 : 2 molar ratio of GroEL and GroES.

984 kDa respectively, which is again in good agreement with the proposed species complex suggested above. However, carrying out a forced fit of the expected molecular masses using DCDT+ for this model (results not shown) gives a relatively poor fit, although some blurring of the peak would be expected if we consider that the $GroEL_{14}$ and $GroES_7$ are in equilibrium between the three-complex system [$GroEL_{14}$, $GroEL_{14}$–$(GroES_7)_1$ and $GroEL_{14}$–$(GroES_7)_2$] as the $GroEL_{14}$ sediments through the sample solvent containing the $GroES_7$ and ATP. Fitting of a third species to the model (Figure 5c) does go some considerable way to improving the fit, although without further investigation we cannot be totally sure of the exact nature of this complex formation.

DISCUSSION

These results strongly indicate that a supply of hydrolysable ATP is necessary for the complex formation between $GroES_7$ and $GroEL_{14}$. There is no evidence from these results of any interaction between these molecules in the absence of nucleotide or when a non-hydrolysable analogue (ATP[S]) is present. Dynamic light-scattering shows no change in hydrodynamic radius when ATP[S] is added to the mixture, but a significant change is observed in the presence of ATP. These results are reinforced by those from sedimentation-velocity analysis. Here, no significant change in sedimentation-coefficient distribution is observed until a supply of ATP is available to the system. These

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findings are in agreement with those of Chandrasekhar and co-workers [14], who also found that the GroEL–GroES complex did not occur in the presence of a non-hydrolysable ATP analogue. This reinforces the case for hydrolysable ATP as a requirement for the chaperonin action of the GroEL/GroES system.

In comparison with these finding, previous workers have suggested that complex formation can occur in the presence of non-hydrolysable ATP analogues. Although only asymmetrical complexes are reported by Llorca and co-workers [11], who found, via electron microscopy, that the distal end of the GroEL cylinder with GroES complexed to one end resembled GroEL devoid of ATP. This finding is backed up by those from X-ray crystallography, where no appreciable conformational change is visualized in the GroEL structure with ATP[S] bound when compared with GroEL in the absence of ATP [26]. These findings therefore indicate that no ring co-operatively takes place in the presence of the non-hydrolysable ATP analogues, which would at least prevent the formation of the 2:1 GroES–GroEL complex. Similarly, Behlke and co-workers [13] suggested 2:1 GroES–GroEL complex formation in the presence of ATP[S] using the hydrodynamic technique of sedimentation equilibrium. However, from the results that they report and from our own sedimentation-equilibrium data, which shows a lowered weight-average molecular mass consistent with no complex formation, we cannot draw the same conclusions. In addition to these findings, only small effects on the stability of the GroEL molecule in the denaturant urea was reported by Lissin [8] in the presence of ATP[S], suggesting that a weak interaction takes place between the ATP analogue and the GroEL molecule. Although the ability of the non-hydrolysable ATP analogues to bind to the GroEL molecule is not disputed here, it appears from these results that conformational changes in the GroEL, and hence the binding of the GroES molecule to the GroEL in the presence of ATP[S], is seriously affected. Our failure to detect any complex formation could have been due to the complex only being present in very small amounts, which may well have been below the resolution of the techniques used here.

In contrast, in the presence of ATP, these results show clear evidence of complex formation, with the ATPase rate of GroEL reduced by over 50% in the presence of GroES, which, if no complex or interaction were taking place, would be unchanged. Photon correlation spectroscopy also shows an ATP concentration-dependency, with mainly 1:1 GroEL–GroES complex stoichiometry at low ATP concentration (0.5 mM) and mainly 2:1 complex formation with higher ATP concentrations (2 mM). This finding is comparable with those of Azem and co-workers [16], who indicated an ATP-concentration-dependency of asymmetrical GroEL–GroES. Similarly, Llorca and co-workers [12] presented evidence of K^+-dependency of the complex formation and a small ATP-concentration-dependency (1–5 μM), for symmetrical complex formation when Mg^{2+} and K^+ levels were kept constant. Correspondingly, sedimentation velocity shows the presence of 2:1 GroES–GroEL complexes in the presence of 2 mM ATP, but only 1:1 complex formation in the presence of ADP, which is again consistent with the finding of Llorca and co-workers [11], and again reinforces the hypothesis that the binding of hydrolysable ATP is a requirement for correct formation of the GroEL–GroES complex.

The evidence presented here clearly suggests that hydrolysable ATP is a requirement for the chaperonin action of the GroEL/GroES system. The unexpected findings, in contrast with those of other workers [13], of no appreciable complex formation in the presence of the non-hydrolysable ATP analogue (ATP[S]) in solution alone adds doubt to previous findings and warrants further investigation into the exact nucleotide requirements of the GroEL–GroES complex formation. The results presented here should offer significant help to others following this path.

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REFERENCES

1 Gething, M. J. and Sambrook, J. (1992) Protein folding in the cell. Nature (London) **355**, 33–45

2 Gibbons, D. L., Hixson, J. D., Hay, N., Lund, P., Gorovits, B. M., Ybarra, J. and Horowitz, P. M. (1996) Intrinsic fluorescence studies of the chaperonin GroEL containing single Tyr-Trp replacements reveal ligand-induced conformational changes. J. Biol. Chem. **271**, 31989–31995

3 Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R. and Saibil, H. R. (1994) Location of a folding protein and shape changes in GroEL–GroES complexes imaged by cryo-electron microscopy. Nature (London) **371**, 261–264

4 Hemmingsen, S. M., Woolford, C. and van der Vies, S. M. (1988) Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature (London) **333**, 330–334

5 Llorca, O., Smyth, M. G., Marco, S., Carrascosa, J. L., Willison, K. R. and Valpuesta, J. M. (1998) ATP binding induces large conformational change in the apical and equatorial domains of the eukaryotic chaperonin containing TCP-1 complex. J. Biol. Chem. **273**, 10091–10094

6 Braig, k., Otwinoski, Z., Hegde, R., Biosvert, D. C., Joachimiak, A. and Horwich, A. L. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. Nature (London) **371**, 578–586

7 Viitanen, P., Gatenby, A. A. and Lorimer, G. H. (1992) Purified chaperonin 60 (GroEL) interacts with the nonnative states of a multitude of *Escherichia coli* proteins. Protein Sci. **1**, 363–369

8 Lissin, N. M. (1995) *In vitro* dissociation and self-assembly of three chaperonin 60s: the role of ATP. FEBS Lett. **361**, 55–60

9 Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, P. D. and Lorimer, G. H. (1990) Chaperonin facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K^+ dependent. Biochemistry **29**, 5665–5671

10 Saibil, H. R., Zheng, D., Roseman, A. M., Hunter, A. S., Watson, G. M. F., Chen, S., auf der Mauer, A., O'Hara, B. P., Wood, S. P., Mann, N. H. et al. (1993) ATP induces large quaternary rearrangements in cage like chaperonin structure. Curr. Biol. **3**, 265–273

11 Llorca, O., Marco, S., Carrascosa, J. L. and Valpuesta, J. M. (1994) The formation of symmetrical GroEL–GroES complexes in the presence of ATP. FEBS Lett. **345**, 181–186

12 Llorca, O., Carrascosa, J. L. and Valpuesta, J. M. (1996) Biochemical characterisation of symmetric GroEL–GroES complexes. J. Biol. Chem. **271**, 68–76

13 Behlke, J., Ristau, O. and Schönfeld, H. J. (1997) Nucleotide dependent complex formation between the *Escherichia coli* chaperonins GroEL and GroES studied under equilibrium conditions. Biochemistry **36**, 5149–5156

14 Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R. and Georgopoulos, C. (1986) Purification and properties of the GroES morphogenic protein of *Escherichia coli*. J. Biol. Chem. **26**, 12414–12419

15 Llorca, O., Pérez-Pérez, J., Carrascosa, J. L., Galán, A., Muga, A. and Valpuesta, J. M. (1997) Effects of the inter-ring communication in GroEL structural and functional asymmetry. J. Biol. Chem. **272**, 32925–32932

16 Azem, A., Diamant, S., Kessel, M., Weiss, C. and Goloubinoff, P. (1995) The protein-folding activity of chaperonins correlates with the symmetric GroEL14–$(GroES7)_2$ heterooligomer. Proc. Natl. Acad. Sci. U.S.A. **92**, 12021–12025

17 Laue, T. M., Shah, B. D., Ridgeway, T. M. and Pelletier, S. L. (1992) Computer-aided interpretation of analytical sedimentation data for proteins. In Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J. and Horton, J. C., eds.), pp. 90–125, Royal Society of Chemistry, Cambridge

18 Philo, J. S. (2000) A method for directly fitting the time derivative of sedimentation velocity data and an algorithm for calculating sedimentation coefficient distribution functions. Anal. Biochem. **279**, 151–163

19 Stafford, III, W. F. (1992) Sedimentation boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. Anal. Biochem. **203**, 295–301

20 Tanford, C. (1961) Transport Processes: Viscosity, J. Wiley and Sons, New York

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21 Garcia de la Torre, J., Carrasco, B. and Harding, S. E. (1997) SOLPRO: theory and computer program for the prediction of SOLution PROperties of rigid macromolecules and bioparticles. Eur. Biophys. J. **25**, 361–372
22 Emes, C. H. and Rowe, A. J. (1978) Frictional properties and molecular weight of native and synthetic myosin filaments from vertebrate skeletal muscle. Biochim. Biophys. Acta **537**, 125–144
23 Boyer, F. R. (1993) Kinetic analysis of an enzyme, tyrosinase. In Modern Experimental Biochemistry, Benjamin Cummings, Menlo Park, CA
24 Harding, S. E. (1999) Photon correlation spectroscopy. In Encyclopaedia of Molecular Biology (Creighton, T. E., ed.), pp. 1847–1849, John Wiley and Sons, New York
25 Xu, Z., Horovitz, A. L. and Sigler, P. B. (1997) The crystal structure of the asymmetric GroEL–GroES–$(ADP)_7$ chaperonin complex. Nature (London) **388**, 741–750
26 Boisvert, D. C., Wang, J. Z. O., Horwich, A. L. and Sigler, P. B. (1996) The 2.4 Å crystal structure of the bacterial chaperonin GroEL complexed with ATP-γ-s. Nat. Struct. Biol. **3**, 170–177

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