Nucleotide-Dependent Complex Formation between the *Escherichia coli* Chaperonins GroEL and GroES Studied under Equilibrium Conditions

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Received November 5, 1996; Revised Manuscript Received February 21, 1997[⊗]

ABSTRACT: Binding of heptameric GroES to the tetradecameric chaperonin GroEL in the absence or presence of nucleotides was investigated by analytical ultracentrifugation. In the absence of nucleotides, the association constant for the binding of GroES to GroEL, K_1, was found to be approximately equal to 3×10^5 M^{-1}. The binding of a second GroES heptamer with only one-fourth the affinity of the first one can be neglected at subequimolecular concentrations relative to GroEL. Under these conditions, mainly an asymmetric "bullet"-shaped complex is formed [see also Schmidt et al. (1994) *Science 265*, 656−659]. In the presence of ADP or ATP analogues such as ATP-γ-S or AMP-PNP, the affinity to bind GroES increases by at least 2 orders of magnitude depending on the nucleotide concentration. With increasing GroES:GroEL ratios in the presence of 1 mM ATP analogue, up to two GroES oligomers were bound to one GroEL oligomer, forming the symmetrical "American football"-shaped complex with apparently high affinity for the first GroES ring and considerably lower for the second one. These are the first results that provide an accurate and quantitative description of the equilibrium between asymmetrical and symmetrical complexes at relatively high concentrations of GroEL and GroES that are proposed to exist *in vivo*. We suggest that the increased affinity of GroEL for GroES plays a role in releasing substrate proteins from the central cavity of GroEL after folding under "non-permissive" conditions.

The bacterial GroE chaperonin system consisting of GroEL and GroES facilitates folding and assembling of non-native proteins *in vivo* as well as *in vitro* (Goloubinoff et al., 1989a,b; Badcoe et al., 1991; Buchner et al., 1991; Fisher, 1992, 1994; Carillo et al., 1992; Wynn et al., 1992; Zhi et al., 1992; Zheng et al., 1993; Miller et al., 1993; Gray & Fersht, 1993). As demonstrated by electron microscopy, GroEL forms a complex of 14 identical 57.3 kDa proteins arranged in 2 stacked heptameric rings (Hendrix, 1979; Hohn et al., 1979; Saibil et al., 1991; Martin et al., 1994). The bacterial GroES appears as a heptameric ring of 10.4 kDa proteins (Chandrasekhar et al., 1986). According to its crystal structure, the co-chaperonin forms a dome of approximately 7.5 nm diameter and 3.0 nm in height with a 0.8 nm orifice in the roof (Hunt et al., 1996). GroEL binds the folding intermediates with low specificity in the central cavity (Vitanen et al., 1992; Jaenicke, 1993). Recently, the three-dimensional structure of GroEL and the binding sites for GroES and non-native proteins have been described on the basis of X-ray crystallographic data (Braig et al., 1994; Fenton et al., 1994). Some of the amino acid residues in the central cavity of GroEL, that are contact residues for GroES, also interact with substrate proteins. Therefore, it is assumed that a competition between both kinds of proteins at the end of the central cavity might occur. GroEL is a weak ATPase. The binding of ATP induces a rotation of parts of the GroEL molecules, especially the apical domain (Saibil et al., 1993; Braig et al., 1994), which facilitates the binding of GroES to GroEL and probably a partial release of the substrate proteins. The steps of ATP binding and its hydrolysis on GroEL in the presence of GroES were described in detail by Todd (Todd et al., 1994). The ATPase activity seems to differ in both heptameric rings of GroEL and depends on the presence of GroES. The co-chaperonin acts as ATPase inhibitor in one toroid and is an inducer of cooperativity for the GroEL ring in the opposite position.

Despite this knowledge and the fact that GroES and MgATP are necessary for release of the substrate proteins from GroEL, details of this mechanism are unknown. Furthermore, it is unclear whether only one or two GroES oligomers are bound to GroEL. Recently, different authors (Azem et al., 1994, 1995; Schmidt et al., 1994a; Harris et al., 1994; Engel et al., 1995; Llorca et al., 1994, 1996) have demonstrated by cross-linking and electron microscopic experiments the formation of both the asymmetrical 1:1 ($GroEL_{14}$−$GroES_7$), or "bullet", complex and the 1:2 [$GroEL_{14}$−$(GroES_7)_2$], or "American football", complex. However, different interpretations were given with respect to conditions under which bullets or footballs exist and for their function in the folding process. Whereas Engel (Engel et al., 1995) favors bullets as productive assemblies, Azem (Azem et al., 1994, 1995) and also Llorca (Llorca et al., 1996) observe protein folding activities also under conditions where symmetrical GroEL−GroES complexes are formed. The data which lead to these conclusions were mostly derived from electron microscopic studies; this technique has the advantage of visualizing such complexes but only permits using one part of the images with a side view for the

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

S0006-2960(96)02755-9 CCC: $14.00

statistical analysis (Engel et al., 1995). Furthermore, different results can be attributed to the glutaraldehyde cross-linking of particles used to stabilize the interaction between GroEL and GroES, which may not always reflect the situation in solution.

Taking into account that GroES and the unfolded proteins bind to the same sites of the central cavity of GroEL, we have to assume a competition reaction between them. To understand the sequestering mechanism, it is useful to know the manner by which GroES is bound to GroEL in the ATP-free and ATP-bound states. Therefore, we have analyzed the heterologous association between GroEL and GroES oligomers under different conditions by studying the sedimentation equilibrium in the analytical ultracentrifuge. This technique allows the analysis of the complex formation of reacting systems under equilibrium conditions without separation of their reactants and complexes. By means of a fitting procedure using the program "Polymole" (Behlke et al., 1995), the partial concentrations, equilibrium constants, and stoichiometries can be calculated from the radial concentration distribution at sedimentation equilibrium considering the initial concentration of reactants and the molecular masses of the species involved. To minimize the possible dissociation reaction of GroES oligomers under very diluted solutions (Zondlo et al., 1995), we have analyzed the interaction with GroEL at concentrations which more closely resemble *in vivo* conditions [see ref 24 in Hayer-Hartl et al., (1995) and Lorimer (1996)].

MATERIALS AND METHODS

The nucleotides ADP, ATP, adenosine 5′-*O*-(3-thiotriphosphate)(ATP-γ-S),[1] and AMP-PNP were purchased from Sigma (Sigma Switzerlands, Buchs).

Isolation and Purification of GroES and GroEL. The chaperonins were isolated and purified following published methods (Buchner et al., 1991) with small modifications described elsewhere (H.-J Schönfeld et al., manuscript in preparation). Briefly, following plasmid overexpression of GroEL or GroES, in *E. coli*, cells were lysed by combined sonication and lysozyme treatment. GroEL was purified by subsequent ammonium sulfate fractionation, anion exchange chromatography (SP-Sepharose, Pharmacia), and gel filtration (Sephacryl S300, Pharmacia). GroES was purified by anion exchange chromatography, dye ligand chromatography (Matrex Green A, Amicon), and gel filtration. The identities of the purified recombinant chaperonins were verified by mass spectroscopy, amino acid analysis, and N-terminal sequencing and were more than 98% homogeneous (estimated by SDS−PAGE) and active in supporting protein folding (Dale et al., 1994). GroEL had ATPase activity (see below).

Polyacrylamide Gel Electrophoresis. Denaturing SDS−PAGE according to the method of Laemmli (1970) was performed under reducing conditions using precast gradient gels (4−20% Tris−glycine gel; Novex, San Diego) running at 150 V for 1.5 h. Gels were stained with Serva Blue R (Serva, Heidelberg). Protein standards were from Novex (Mark 12).

[1] Abbreviations: ATP-γ-S, adenosine 5′-*O*-(3-triphosphate); AMP-PNP, adenosine 5′-(β,γ-iminotriphosphate); PAGE, polyacrylamide gel electrophoresis.

Analysis for ATP and ADP in Preparations of Purified GroEL and GroES. ATP and ADP were analyzed following a published procedure (Schultz et al., 1993). Briefly, ATP was determined by measuring the ATP-dependent oxidation of luciferin catalyzed by luciferase in an bioluminometric assay. No ATP was detected in any chaperonin. When GroEL was supplemented with external ATP, ATP values were found to decrease in a time-dependent manner, demonstrating the ATPase activity of GroEL.

ADP, possibly present in the purified chaperonins, was converted to ATP by pyruvate kinase and then determined as described above. No ADP was detected in purified GroEL or GroES by this method. The sensitivity of the assay was higher than 1 mol nucleotide per 10 mol of chaperonin for both nucleotides, as verified by spike experiments.

Molecular Mass Determination. Molecular masses (M) of the chaperonins were determined by means of an XL-A analytical ultracentrifuge (Beckman). The values were obtained either from sedimentation and diffusion experiments at 20 °C using a synthetic boundary cell or by the sedimentation equilibrium technique at 10 °C. Diffusion coefficients (D) were calculated from the time-dependent boundary spreading of the concentration gradient at 4000 or 6000 rpm measured at different wavelengths. From the moving boundary in highspeed experiments, sedimentation coefficients (s) were determined using a modified Svedberg program (Philo, 1994) or the program LAMM (Behlke & Ristau, 1997) which permits the simultaneous estimation of sedimentation and diffusion coefficients. Partial specific volumes ($\bar{\nu}$) were calculated based on amino acid composition and density increments of the amino acids (Cohn & Edsall, 1943). In addition to the molecular mass obtained by the Svedberg equation, the determined parameters also allow the calculation of the volume (V) by eq 1

$$V = M\bar{\nu}/N_A \tag{1}$$

or the frictional ratio by eq 2

$$f/f_0 = 10^{-8}\left(\frac{1-\rho\bar{\nu}}{D^2 s\bar{\nu}}\right)^{1/3} \tag{2}$$

Here ρ means the solvent density. Assuming spheres for single proteins or their subunits, the radius of unhydrated proteins can be calculated by eq 3:

$$r = \left(\frac{3V}{4\pi}\right)^{1/3} \tag{3}$$

In solution, the species are larger by about 0.3 nm (water shell). The friction of protein-bound water with the bulk water decreases the hydrodynamic mobility, resulting in lower sedimentation and diffusion coefficients. This has to be considered when constructing models composed of spheres for the calculation of theoretical sedimentation and diffusion coefficients using the program "Hydro" (Garcia de la Torre et al., 1994).

Sedimentation equilibrium experiments were carried out in six-channel cells. Each compartment of these cells was filled with 80 μL of GroEL, GroES, or different mixtures of both proteins. The samples were centrifuged 2 h at 8000 rpm (overspeed technique) followed by an equilibrium speed of 6000 rpm for 30−40 h at 10 °C. In some experiments,

the time to attain the sedimentation equilibrium was extended up to 50 or 60 h without any significant changes. Radial concentration distributions were recorded at three different wavelengths, in most cases 280, 285, and 290 nm.

The molecular masses (M) of single proteins were obtained by fitting the radial concentration distribution according to eq 4

$$c_{(r)} = c_0 e^{MF} \tag{4}$$

with

$$F = [(1 - \rho\bar{\nu}_i)\omega^2(r^2 - r_0{}^2)]/2RT \tag{5}$$

Here $\bar{\nu}_i$ is the partial specific volume of component i, ω the angular velocity, R the gas constant, and T the absolute temperature.

Complex Formation between GroEL and GroES. Complex formation between different macromolecules can be easily derived from the molecular mass determinations when associates are formed with high affinity, $K_a > 10^8$ M^{-1}. Because most of the complexes are of lower affinity, we have to estimate the association constants of such an interacting system by fitting the sum of exponential functions, given in eq 6, to the experimentally obtained radial scanning curves (A_r) according to Behlke et al. (1995):

$$A_r = \epsilon_R c_R e^{BM_R F} + \epsilon_L c_L e^{M_L F} + c_R \sum_{j=1}^{n} (\epsilon_R + j\epsilon_L) c_L^j K_j e^{(BM_R + jM_L)F} \tag{6}$$

Here ϵ_R, ϵ_L, c_R, and c_L are the extinction coefficients and free concentrations of the receptor molecule (R = GroEL) or the free ligand concentration (L = GroES) at the radial position r_0, respectively. B means the difference in buoyancy between R and L, j the number of possible binding steps, and K_j the binding constant corresponding to the binding step. To get more reliable data for the estimated parameters, we have to reduce the number of variables describing the binding reaction. This can be achieved (i) by separate determination of molecular masses M_R and M_L; (ii) by using a statistical binding model for equal binding sites according to Wyman and Gill (1990); and (iii) by considering the mass conservation. In each experiment, three absorbance profiles obtained at three different wavelengths were analyzed. This allowed us to determine the total concentration of the components in an arbitrary sector by numerical integration. Using all three profiles together improved the accuracy of the data. According to the statistical binding model, we can replace K_j by $(1/n^j)\binom{n}{j}K_1{}^j$, where K_1 is the binding constant for the first step. If we substitute $(1/n^j)\binom{n}{j}$ with $G(j)$ and $1/K_1$ by K_d or $c_L K_1 = x$, eq 6 can be modified as follows:

$$A_r = \epsilon_R c_R e^{BM_R F} + \epsilon_L K_d x e^{M_L F} + c_R \sum_{j=1}^{n} (\epsilon_R + j\epsilon_L) G(j) x^j e^{(BM_R + jM_L)F} \tag{7}$$

For the total concentrations c_{Rt} and c_{Lt}, the integration of the model function results in eqs 8 and 9:

$$c_{Rt}(r_b - r_m) = c_R\left[\int_{r_m}^{r_b} e^{BM_R F}\, dr + \sum_{j=1}^{n} x^j G(j) \int_{r_m}^{r_b} e^{(BM_R + jM_L)F}\, dr\right] \tag{8}$$

$$c_{Lt}(r_b - r_m) = K_d x \int_{r_m}^{r_b} e^{M_L F}\, dr + c_R \sum_{j=1}^{n} j x^j G(j) \int_{r_m}^{r_b} e^{(BM_R + jM_L)F}\, dr \tag{9}$$

with r_b and r_m the radius positions at the cell bottom and the meniscus, respectively.

The substitution of c_R and K_d by functions of eqs 8 and 9 enables us to reduce the estimated parameters to only c_L. Three of the absorbance profiles (eq 6 or eq 7 represents such a profile only for one wavelength) were simultaneously fitted (global) by nonlinear regression using the program "Polymole" which was successfully applied earlier in the analysis of other complexes (Behlke et al., 1994, 1995; Grunau et al., 1995). The optimal fit to the radial distribution curves allows us to estimate the association constant(s) of the complex(es). An independent possibility to analyze the stoichiometry of an interacting system is based on the usage of eq 10 (Behlke et al., 1995);

$$A_r = \epsilon_R c_R e^{BM_R F} + \epsilon_L c_L e^{M_L F} + c_R (c_L K)^j (\epsilon_R + j\epsilon_L) e^{(BM_R + jM_L)F} \tag{10}$$

This model function can be employed for experiments with excess ligand concentration. It considers only one type of complex and includes the mass conservation in analogy to eqs 8 and 9. By fitting eq 10 to the radial concentration distribution, the occupation number, j, can be treated as a normal parameter with arbitrary values. Its estimation can be performed as usual. By means of the partial concentrations (c_i) obtained from the fitting procedures and the molecular masses (M_i) for the reactants and complexes we can calculate the weight-average molecular mass M_w using eq 11:

$$M_w = \frac{\sum_i c_i M_i}{\sum_i c_i} \tag{11}$$

for the reacting GroEL/S system which considers all the components involved.

RESULTS

Characterization of GroEL and GroES. The molecular masses of both oligomeric proteins were determined either by sedimentation diffusion experiments or by sedimentation equilibrium runs (Table 1). Both methods result in molecular mass data in agreement with a tetradecameric structure for GroEL and a heptameric structure for GroES. These proteins form stable oligomers under the conditions chosen for the ultracentrifugation experiments.

The frictional ratios f/f_0 of the two molecules (Table 1) indicate the gross conformation of a macromolecule in solution. The considerably higher value of 1.44 for GroES compared with 1.30 for GroEL is in agreement with the flat ring structure of about 8 nm diameter and 3 nm height and

Table 1: Hydrodynamic Data for GroEL and GroES[a]

	$s_{20,w}$ (S)	$D_{20,w} \times 10^7$ cm^2/s	$\bar{\nu}$ (mL/g)	$M_{s,D}$ (kDa)	M_{eq} (kDa)	f/f_0
GroEL	22.13 ± 0.16	2.59 ± 0.09	0.747_8	802.6 ± 16	795.8 ± 12	1.30
GroES	3.92 ± 0.04	5.30 ± 0.14	0.751_0	72.4 ± 2.1	71.9 ± 2.3	1.44

[a] $M_{s,D}$ and M_{eq} are molecular mass values either from sedimentation and diffusion coefficients or from sedimentation equilibria.

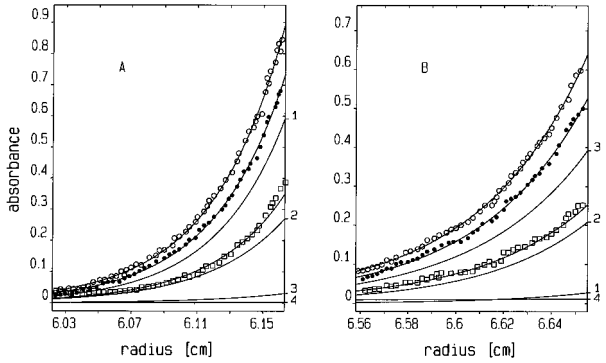

FIGURE 1: Radial absorbance scans at 280 nm (○), 285 nm (●), and 290 nm (□) from cells containing 0.71 μM GroEL and 1.59 μM GroES in the presence of 1 mM ATP-γ-S (A) and in the absence of nucleotides (B) in 50 mM Tris-HCl, pH 7.7, 100 mM KCl, and 10 mM $MgCl_2$ at 10 °C. The solid curves represent the best simultaneous fits of the 1:2 association (1) and a 1:1 association complex (2) of GroEL/GroES as well as the free reactants GroEL (3) and GroES (4).

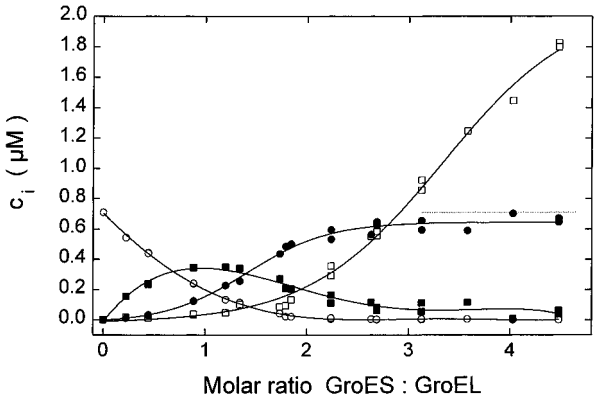

FIGURE 2: Partial concentrations of GroEL−GroES (■), GroEL−$GroES_2$ (●), free GroEL (○), and free GroES (□) at increasing GroES:GroEL molar ratios at 10 °C and in the presence of 1 mM ATP-γ-S. The GroEL concentration was kept constant at 0.71 μM in 50 mM Tris-HCl, pH 7.7, 100 mM KCl, and 10 mM $MgCl_2$. The dotted line represents the highest possible partial concentration of the symmetrical complex.

was verified by modeling using the program "Hydro" (Garcia de la Torre et al., 1994), which requires the spherical coordinates and radii of the spheres representing the monomers in the assemblies. In contrast, the GroEL cylinder with a diameter of 14 nm and a height of 13 nm was found to deviate less from a sphere.

The addition of ATP-γ-S alone did not change the molecular mass of GroEL significantly (data not shown). The changes observed in molecular mass when nucleotide and GroES are added to GroEL can therefore be attributed to formation of GroEL−GroES complexes.

Interaction between GroEL and GroES. To study the complex formation, different mixtures of GroEL and GroES were centrifuged under conditions described under Materials and Methods. Analysis of the data using the computer program "Polymole" yielded the binding constants and corresponding partial concentrations of complexes as well as free reactants (Figure 1). Most of the GroEL (loading concentration ~0.71 μM) in the nucleotide-free solution (Figure 1B) remains in the uncomplexed state (~60%). About 30% of GroEL forms a 1:1 complex, and a negligibly small amount of the chaperone bound two GroES. In the presence of 1 mM ATP-γ-S (Figure 1A), high proportions of 1:2 and 1:1 complexes are formed, and only small amounts of reactants remained unbound.

When adding increasing amounts of GroES to a 0.71 μM solution of GroEL in the absence of nucleotides (data not shown), a slight tendency to form heterologous complexes was observed. Most of the added GroES remained free, and only low amounts of complexes were formed with GroEL. However, in the presence of 1 mM ATP-γ-S (Figure 2), already at low concentrations of GroES most of it was bound to GroEL. At an equimolar ratio of both proteins, about 50% of GroEL formed a 1:1 complex, but about a 25% of GroEL bound up 2 mol of GroES. The residual 25% of the amount of GroEL remained in the unbound state. In solutions containing a 2-fold molar excess of GroES relative to GroEL, a far higher amount of GroEL was occupied by two GroES. Further addition of GroES led to almost complete formation of a complex containing one GroEL and two GroES. When eq 10 was fitted to the experimental curves of the highest measured ratio of GroES:GroEL, the occupation number j was calculated to between 1.7 and 1.8.

Engel (Engel et al., 1995) proposed that only asymmetrical 1:1 complexes of GroEL and GroES are formed at pH 7.2 in the presence of 5 mM $MgCl_2$, whereas symmetrical 1:2 complexes would only be formed at a higher pH of 8.0 and at the higher $MgCl_2$ concentration of 50 mM. We carried out sedimentation equilibrium runs under both conditions, but observed (i) no significant difference in the interaction of GroEL and GroES (Figure 3A,B), (ii) more than one co-chaperonin was complexed with GroEL in the presence of 1 mM ATP-γ-S or AMP-PNP, and (iii) the complex formation was only affected by the GroES:GroEL ratio.

When analyzing a solution containing a 2.2-fold molar excess of GroES over GroEL (loading concentration 1.59 μM GroES and 0.71 μM GroEL) in the presence of increasing concentrations of ATP-γ-S, we observed a decrease of the free proteins starting at a nucleotide concentration of 10 μM. Due to the negative cooperativity with respect to nucleotide between the rings of GroEL, only one ring of GroEL is populated with GroES at these low ATP-γ-S concentrations. As the ATP-γ-S concentration increases, and the second of GroEL becomes populated with nucleotide, formation of the symmetric GroEL−$GroES_2$ complex becomes apparent. Complex formation increased further and was found completed at a nucleotide concentration of about 1 mM (Figure 4).

The program "Polymole" enables us to calculate the association constants and partial concentrations of complexes and free reactants for the reacting system (Figure 5). In the absence of the ATP analogue ATP-γ-S, the first GroES was

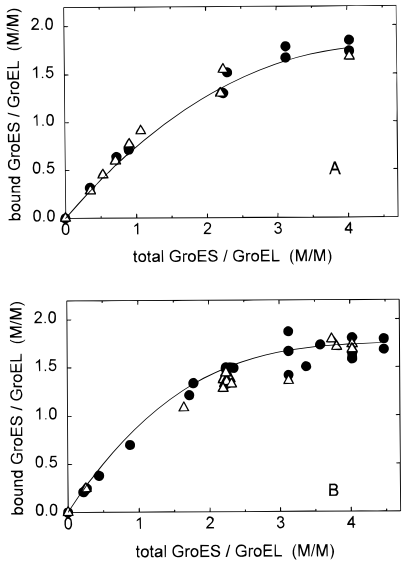

FIGURE 3: Analysis of the interaction between GroEL and GroES in two previously published conditions. Different amounts of GroES were mixed with 0.71 μM GroEL as indicated by the GroES:GroEL molar ratios in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 50 mM $MgCl_2$ (△) or in 20 mM MOPS−KOH, pH 7.2, 50 mM KCl, and 5 mM $MgCl_2$ (●) in the presence of 1 mM AMP-PNP (A) or 1 mM ATP-γ-S (B).

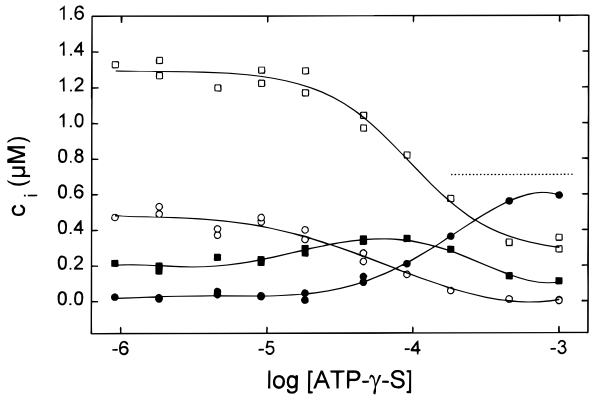

FIGURE 4: Influence of increasing concentrations of ATP-γ-S in a mixture of 0.71 μM GroEL and 1.59 μM GroES on the partial concentration of GroEL−GroES (■), GroEL−$GroES_2$ (●), free GroEL (○), and free GroES (□). Conditions were as described in Figure 2. The dotted line represents the highest possible partial concentration of the symmetrical complex.

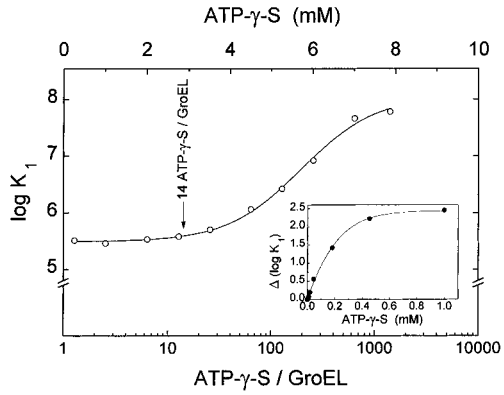

FIGURE 5: Influence of the molar ATP-γ-S:GroEL ratio on the association constant K_1. Insert: ATP-γ-S-dependent increase of K_1.

bound to GroEL with moderate affinity. The association constant K_1 was found to be approximately equal to 3×10^5 M^{-1}. As expected from the nucleotide-induced affinity

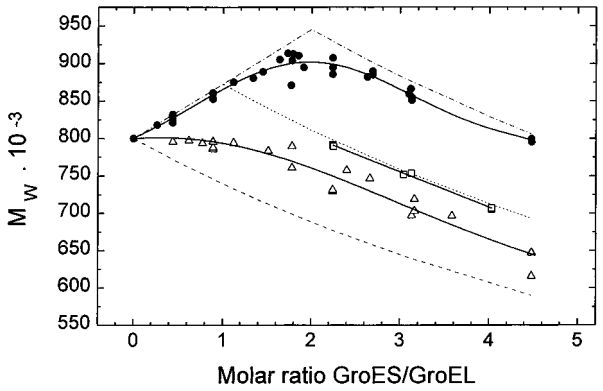

FIGURE 6: Influence of GroES:GroEL ratio on complex formation demonstrated by the calculated weight-average molecular masses. The data were obtained in the absence of nucleotide (△), or in the presence of 1 mM ADP (□) or 1 mM ATP-γ-S (●), respectively. The dashed and dotted lines from bottom to top correspond to theoretical weight-average molecular masses when zero, one, or two GroES bind to GroEL. Loading concentrations were 0.71 μM GroEL and 1.59 μM GroES. Conditions were as described in Figure 2.

of GroEL to bind GroES and the data presented and from data in Figure 4, the association constants K_1 increased by 2 orders of magnitude with increasing concentration of ATP-γ-S. The nucleotide concentration which induced a 50% increase in the affinity of GroEL for GroES was found to be about 180 μM; the effects of the nonhydrolyzable ATP analogue AMP-PNP were found to be similar to those of ATP-γ-S (data not shown).

We fitted the absorption profiles derived from the experiments shown in Figure 2 by eq 7 and assumed that both binding sites in the central cavity of GroEL were equal. From the statistical point of view, the affinity for the second binding step is expected to be reduced to one-fourth of that of the first one (Wyman & Gill, 1990). On the other hand, when we used a model function like eq 6 [see also Johnson et al. (1981)] with arbitrary (nonequal) binding sites to fit the experimental curves and inserted the initial values as obtained by eq 7, the obtained result did not differ significantly. This indicates that each binding site has indeed equal or at least similar affinity.

The experimentally derived partial concentrations, c_i, of GroEL and GroES in the free and complexed state were used to calculate the weight-average molecular masses, M_w, at different concentrations of AMP-PNP and a fixed GroES:GroEL molar ratio of about 2.2 using eq 11 (data not shown). In the absence of nucleotides, the M_w is about 720 kDa, considerably smaller than the mass of GroEL, reflecting the large amount of unbound GroES. In contrast, in the presence of 1 mM ATP-γ-S or AMP-PNP, almost two GroES bind to one GroEL (data not shown).

When increasing amounts of GroES were added to GroEL in the presence of 1 mM ATP-γ-S, the apparent weight-average molecular masses increased significantly up to about 900 kDa and decreased again when the GroES:GroEL molar ratio in the sample exceeded 2 (Figure 6). When GroES was added in the absence of nucleotides, the M_w values continuously decreased because most GroES remained in an unbound state. However, when increasing amounts of GroES were added to GroEL in the presence of 1 mM ADP, approximately one co-chaperonin is bound by association constants of $(3-7) \times 10^7$ M^{-1}. At a 3−4-fold molar excess

of GroES, the stoichiometry of the complex did not deviate significantly from 1:1 (Figure 6), indicating that the binding of a second GroES to the GroEL cylinder is strongly inhibited in the presence of ADP.

DISCUSSION

We have analyzed the GroE chaperonin system by analytical ultracentrifugation, to understand their quaternary structures and their interaction in solution. Sedimentation velocity and sedimentation equilibrium experiments performed with GroEL and GroES at micromolar concentrations close to those *in vivo* confirmed that the isolated components existed exclusively as tetradecameric and heptameric molecules, respectively. At considerably lower protein concentrations such as those which have been used previously (Holl-Neugebaueret al., 1991; Langer et al., 1992), the oligomers, especially GroES, are less stable (Zondlo et al., 1995). Under our conditions, the oligomeric state of GroEL did not change in the presence of the ATP analogue ATP-γ-S. Based on these results, we considered tetradecamers and heptamers as the smallest molecular entities of GroEL and GroES, respectively, when modeling the sedimentation profiles that we obtained from mixtures of both chaperonins.

In the absence of nucleotides (Figure 5), there was a weak association between GroEL and GroES with an association constant, K_1, approximately equal to 3×10^5 M^{-1} for the asymmetrical (1:1) complex. GroEL has two binding sites that can be occupied independently, and we therefore have to assume statistical binding of a second GroES molecule. However, the affinity of the second GroES is reduced to about one-fourth of that of the first one (Wyman & Gill, 1990), and a symmetrical (1:2) complex was apparently absent under these conditions. We note that in contrast to our interpretation, Engel and co-workers considered the weak GroEL−GroES interactions they observed in equilibrium dialysis experiments in the absence of nucleotides to be nonspecific [see Figure 2 in Engel et al. (1995)]. The possibility that GroES monomers are bound to GroEL as substrate is very unlikely because of the considerable stability of GroES heptamers at the higher concentrations used in the experiments [see also Zondlo et al. (1995)].

In the presence of the nonhydrolyzable ATP analogues AMP-PNP and ATP-γ-S (Figures 2 and 5), we observed an increase of K_1 by more than 2 orders of magnitude ($K_1 = 8 \times 10^7$ M^{-1}), probably induced by conformational changes in the apical domains of GroEL (Braig et al., 1994; Fenton et al., 1994). At low up to equimolar GroES:GroEL ratios, asymmetrical complexes were formed while at higher molar ratios symmetrical complexes were observed. Based on electron microscopic images, such complexes have previously been described as "bullets" and "American footballs", respectively (Azem et al., 1994; Schmidt et al., 1994a; Harris et al., 1994; Engel et al., 1995; Llorca et al., 1996). We found binding of GroES to GroEL saturated at a molar ratio of about 3:1 and at a nucleotide concentration of about 1 mM. By varying the nucleotide concentration, the first increase of K_1 occurred at a molar nucleotide:GroEL ratio of about 14, indicating a cooperative structural change after the binding of one nucleotide per subunit of GroEL.

Some of our results that differed from published results concerning GroEL/GroES interactions may be explained by the lower protein concentrations used in those experiments (Engel et al., 1995; Fisher & Yuan, 1994; Schmidt et al., 1994b; Weissman et al., 1994), probably leading to more or less extensive dissociation of the oligomeric reactants.

To our knowledge, our results provide the first reliable qualitative and quantitative description of asymmetrical and symmetrical GroEL−GroES complexes under different conditions in solution. For such measurements, the analytical ultracentrifugation method has several advantages compared with other techniques. It allows the quantitative determination of the partial concentrations of free reactants and complexes in solution without calibration by standards and without limitations, inherent in electron microscopic analysis. In electron microscopy, only special side views of chaperonin particles can be considered for statistical analysis (Engel et al., 1995). Furthermore, cross-linking with glutaraldehyde, which was used for stabilization of complexes for electron microscopic studies (Engel et al., 1995), is not necessary for the sedimentation equilibrium technique that involves only relatively low centrifugation forces. Chemical modification induced by cross-linking may create artifacts (Peters & Richards, 1977) and was avoided in our experiments. Moreover, in contrast to separation methods such as gel filtration chromatography, in sedimentation equilibrium experiments reactants and complexes exist side-by-side, forming a real equilibrium without disturbances. The program "Polymole" permits the direct estimation of the association constant(s) without disturbing the complex formation or equilibria. At present, the most precise data for the binding of more than one ligand to a receptor protein can be derived assuming equal binding sites (see Materials and Methods). Additional information about the stoichiometry of the reaction can be obtained, based on considerations of the conservation of mass.

In general, our findings with respect to the appearance of symmetrical structures as a function of the molar GroES:GroEL ratio are in very good agreement with data recently published by Azem [Figure 2 in Azem et al., (1995)]. Although these authors used chemical cross-linking in their studies, cross-linking apparently occurred fast enough [higher temperature and glutaraldehyde concentration than used by others (Engel et al., 1995)] to obtain a snapshot of the equilibria without disturbances caused by chemical modification of the components.

We observed no differences in complex formation at pH 7.2 compared with pH 8.0 or at low magnesium concentrations (5 mM $MgCl_2$) compared with high magnesium concentrations (50 mM $MgCl_2$) (Figure 3). This also confirms recent data obtained by fluorescence measurements (Török et al. 1996). Different results (Engel et al., 1995) may have been related to the lower chaperone concentrations used in electron microscopic studies (0.12 μM GroEL) and especially equilibrium dialysis (60 nM GroEL), or by the limitations of the methodology discussed above.

Recently, the kinetic constants k_{on} and k_{off}, obtained from surface plasmon resonance spectroscopy (SPR), were used to calculate the association constants of GroEL−GroES complexes (Hayer-Hartl et al., 1995; Murai et al., 1995). Interestingly, the affinity constants deduced from the SPR experiments are on the average 2 orders of magnitude higher than those described here. As pointed out by Schuck (1996) and others (Edwards et al., 1995; Nieba et al., 1996), the SPR method, although technically elegant, has the disad-

vantage that measurements are done in a dextran matrix and not in free solution, which might strongly influence the results. There is the limitation of transport in SPR experiments that influences the kinetics (Schuck, 1996) with the consequence that only apparent and not absolute association or dissociation rate constants can be measured. Furthermore, such high association constants as deduced from SPR experiments [up to 10^{10} M^{-1} (Hayer-Hartl et al., 1995)] are comparable to those of antigen−antibody interactions, and it would require extraordinarily high amounts of energy for the cell to effect repeated association and dissociation as proposed for GroEL−GroES during the protein folding reaction (Martin et al., 1993).

The GroEL−GroES chaperonin system is involved in protein folding under "non-permissive" conditions where unassisted spontaneous folding could not occur (Schmidt et al., 1994b). In the presence of GroES and GroEL, folding rates are comparable or sometimes faster than the rate of spontaneous folding (Todd et al., 1994; Peralta et al., 1994). GroES and the protein being folded must compete for sites within the central cavity of GroEL (Fenton et al., 1994). In order to release substrate intermediates from GroEL efficiently, their affinities must be considerably lower than GroES. Conceivably, there is a decreased interaction between the assisted proteins and GroEL induced by ATP. In the presence of ATP, the central cavity of GroEL is widened (Braig et al., 1994), and presumably its interactions with the substrate protein are weakened. On the other hand, our data clearly show that ATP analogues induce an affinity enhancement for the GroEL−GroES interaction of more than 2 orders of magnitude. Taken together, both mechanisms may explain the exchange of folding intermediates by GroES and MgATP (Weissman et al., 1995).

The concentration of GroEL in the *E. coli* cell was calculated as 2.6 μM and the GroES:GroEL molar ratio as 2:1 (Lorimer, 1996), whereas the ATP concentration is about 8 mM (Llorca et al., 1996). Based on these concentrations and due to the general observation that in equilibria of different oligomeric states of proteins these are shifted toward the higher oligomeric forms at higher protein concentrations, we conclude from our data that mainly symmetrical GroEL−$GroES_2$ complexes are present under *in vivo* conditions. However, the observed equilibria also imply that much lower amounts of asymmetrical GroEL−GroES complexes are always present. The question of whether only one structure is an inactive reservoir of chaperones and the other is active in protein folding or if both structures are active in different steps of protein folding has to be answered by experiments that focus on the dynamics of the GroE systems. From the present point of view, the footballs should be considered as intermediates during the chaperonin-mediated protein folding cycle (Török et al., 1996). Using analytical ultracentrifugation, we are currently analyzing the influence of substrate proteins on the association of GroEL and GroES.

ACKNOWLEDGMENT

The skillful technical assistance of Mrs. Bärbel Bödner is gratefully acknowledged. We thank Mr. Bernd Pöschl for his technical help in protein purification. Furthermore, we thank Dr. Peter Schuck for valuable discussions and Dr. Howard Etlinger for reading the text.

REFERENCES

Azem, A., Kessel, M., & Goloubinoff, P. (1994) *Science 265*, 653−665.
Azem, A., Diamant, S., Kessel, M., Weiss, C., & Goloubinoff, P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12021−12025.
Badcoe, I. G., Smith, C. J., Halsall, D. J., Holbrook, J. J., Lund, P., & Clarke, A. R. (1991) *Biochemistry 30*, 9195−9200.
Behlke, J., & Ristau, O. (1997) *Biophys. J. 72*, 428−434.
Behlke, J., Ristau, O., & Knespel A. (1994) *Prog. Colloid Polym. Sci. 94*, 40−45.
Behlke, J., Ristau, O., & Marg, A. (1995) *Prog. Colloid Polym. Sci. 99*, 63−68.
Bochkareva, E. S., Lissin, N. M., Flynn, G. C., Rothman, J. E., & Girshovich A. S. (1992) *J. Biol. Chem. 267*, 6796−6800.
Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., & Sigler, P. B. (1994) *Nature 371*, 578−586.
Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X., & Kiefhaber, T. (1991) *Biochemistry 30*, 1586−1591.
Carrillo, N., Ceccarelli, E. A., Krapp, A.-R., Boggio, S., Ferreyra, R. G., & Viale, A. M. (1992) *J. Biol. Chem. 267*, 15537−15541.
Chandrasekhar, C. N., Tilly, K., Woolford, C., Hendrix, R., & Georgopoulos, C. (1986) *J. Biol. Chem. 261*, 12414−12419.
Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R., & Saibil, H. R. (1994) *Nature 371*, 261−264.
Cohn, E. J., & Edsall, J. T. (1943) *Proteins, amino acids and peptides*, Academic Press, New York.
Dale, G. E., Schönfeld, H.-J., Langen, H., & Stieger, M. (1994) *Protein Eng. 7*, 925−931.
Edwards, P. R., Gill, A., Pollard-Knight, D. V., Hoare, M., Buckle, P. E., Lowe, P. A., & Leatherbarrow, R. J. (1995) *Anal. Biochem. 231*, 210−217.
Engel, A., Hayer-Hartl, M. K., Goldie, K. N., Pfeifer, G., Hegerl, R., Müller, S., da Silva, A. C. R., Baumeister, W., & Hartl, F. U. (1995) *Science 269*, 832−836.
Fenton, W. A., Kashi, Y., Furtak, K., & Horwich, A. L. (1994) *Nature 371*, 614−619.
Fisher, M. T. (1992) *Biochemistry 31*, 3955−3963.
Fisher, M. T. (1994) *J. Biol. Chem. 269*, 13629−13636.
Fisher, M. T., & Yuan, X. (1994) *J. Biol. Chem. 269*, 29598−29601.
Garcia de la Torre, J., Navarro, S., Lopez-Martinez, M. C., Diaz, F. G., & Lopez-Cascales, J. J. (1994) *Biophys. J. 67*, 530−531.
Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989a) *Nature 337*, 44−47.
Goloubinoff, P., Christeller, J. T., Gatenby, A. A., & Lorimer, G. H. (1989b) *Nature 342*, 884−888.
Gray, T. E., & Fersht A. R. (1991) *FEBS Lett. 292*, 254−258.
Gray, T. E., & Fersht, A. R. (1993) *J. Mol. Biol. 232*, 1197−1207.
Grunau, C., Dettmer, R., Behlke, J., & Bernhardt, R. (1995) *Biochem. Biophys. Res. Commun. 210*, 1001−1008.
Harris, J. R., Plückthun, A., & Zahn, R. (1994) *J. Struct. Biol. 112*, 216−230.
Hayer-Hartl, M. K., Martin, J., & Hartl, F. U. (1995) *Science 269*, 836−841.
Hendrix, R. W. (1979) *J. Biol. Chem. 129*, 375−392.
Hohn, T., Hohn, B., Engel, A., & Wurts, M. (1979) *J. Biol. Chem. 129*, 359−373.
Holl-Neugebauer, B., Rudolph, R., Schmidt, M., & Buchner, J. (1991) *Biochemistry 30*, 11609−11614.
Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L., & Deisenhofer, J. (1996) *Nature 379*, 37−45.
Jackson, G. S., Staniforth, R. A., Halsall, D. J., Atkinson, T., Holbrook, J. J., Clark, A. R., & Burston, S. G. (1993) *Biochemistry 32*, 2554−2563.
Jaenicke, R. (1993) *Curr. Opin. Struct. Biol. 3*, 104−112.
Johnson, L. M., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575−588.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Langer, T., Pfeiffer, G., Martin, J., Baumeister, W., & Hartl, F. U. (1992) *EMBO J. 11*, 4757−4765.
Llorca, O., Marco, S., Carrascosa, J. L., & Valpuesta J. M. (1994) *FEBS Lett. 345*, 181−185.

Llorca, O., Carrascosa, J. L., & Valpuesta J. M. (1996) *J. Biol. Chem. 271*, 68−76.
Lorimer, G. H. (1996) *FASEB J. 10*, 5−9.
Martin, J., Mayhew, M., Langer, T., & Hartl, F. U. (1993) *Nature 366*, 228−233.
Martin, J., Goldie, K. N., Engel, A., & Hartl, F. U. (1994) *Biol.-Chem. Hoppe Seyler 375*, 635−639.
Miller, A. D., Maghlaoui, K., Albanese, G., Kleinjan, D., & Smith, C. (1993*) Biochem. J. 291*, 139−144.
Murai, N., Taguchi, H., & Yoshida, M. (1995) *J. Biol. Chem. 270*, 19957−19963.
Nieba, L., Krebber, A., & Plückthun, A. (1996) *Anal. Biochem. 234*, 155−165.
Peralta, D., Hartman, D. J., Hoogenraad, N. J., & Hoj, P. B. (1994) *FEBS Lett. 339*, 45−49.
Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 523−551.
Philo, J. S. (1994) in *Modern Analytical Ultracentrifugation* (Schuster, T. M., & Laue, T. M., Eds.), pp 156−170, Birkhäuser, Boston.
Saibil, H., & Wood, S. (1993) *Curr. Biol. 3*, 207−213.
Saibil, H. R., Dong, S., Wood, S., & Auf Der Mauer, A. (1991) *Nature 353*, 25−26.
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., Barnett, L. K., & Ellis, R. J. (1993) *Curr. Opin. Struct. Biol. 3*, 265−273.
Schmidt, M., Rutkat, K., Rachel, R., Pfeifer, G., Jaenicke, R., Viitanen, P. V., Lorimer, G. H., & Buchner, J. (1994a) *Science 265*, 656−659.
Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H., & Viitanen, P. V. (1994b) *J. Biol. Chem. 269*, 10304−10311.
Schuck, P. (1996) *Biophys. J. 70*, 1230−1249.
Schultz, V., Sussman, I., Bokvist, K., & Tornheim, K. (1993) *Anal. Biochem. 215*, 302−320.
Todd, M. J., Viitanen, P. V., & Lorimer, G. H. (1994) *Science 265*, 659−666.
Török, Z., Vigh, L., & Goloubinoff, P. (1996) *J. Biol. Chem. 271*, 16180−16186.
Viitanen, P. V., Gatenby, G. H., & Lorimer, G. H. (1992) *Protein Sci. 1*, 363−369.
Weissman, J. S., Kashi, Y., Fenton, W. A., & Horwich, A. L. (1994) *Cell 78*, 693−702.
Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., & Horwich, A. L. (1995) *Cell 83*, 577−587.
Wyman, J., & Gill, S. J. (1990) *Binding and Linkage*, University Science Books, Mill Valley, CA.
Wynn, R. M., Davie, J. R., Cox, R. P., & Chuang, D. T. (1992) *J. Biol. Chem. 267*, 12400−12403.
Zheng, X., Rosenberg, L. E., Kalousek, K., & Fenton, W. A. (1993) *J. Biol. Chem. 268*, 7489−7493.
Zhi, W., Landry, S. J., Gierasch, L. M., & Srere, P. A. (1992) *Protein Sci. 1*, 522−529.
Zondlo, J., Fisher, K. E., Lin, Z., Ducote, K. R., & Eisenstein E. (1995) *Biochemistry 34*, 10334−10339.

BI962755H