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The Structural Stability of the Co-chaperonin GroES

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The structural stability of the co-chaperonin GroES has been studied by high sensitivity differential scanning calorimetry and circular dichroism under different solvent conditions. The thermal folding/unfolding of GroES is a spontaneous reversible process involving a highly cooperative transition between folded heptamers and unfolded monomers. During the denaturation process folded monomers are energetically unfavorable and consequently never become populated to an appreciable degree. Analysis of the high resolution structure indicates that isolated folded monomers of GroES bury a significantly smaller fraction of their total surface than typical globular proteins of similar molecular mass. For this reason the intramolecular interactions within each GroES monomer appear not to be sufficient for thermodynamic stabilization. The stabilization of the heptameric structure is due primarily to intersubunit interactions rather than intrasubunit interactions. These interactions favor oligomerization both enthalpically and entropically. Despite the high density of charged residues, the stability of GroES shows no measurable dependence on salt concentration at pH 7. On the other hand, millimolar concentrations of magnesium stabilize GroES, presumably by specific binding. The stabilization elicited by Mg^{2+} is consistent with a dissociation constant of the order of 0.5 mM and approximately three binding sites per heptamer. These results emphasize the role of quaternary structure in the stabilization of small oligomeric proteins.

Keywords: GroES; chaperonins; protein folding; thermodynamics; calorimetry

Introduction

Heat shock proteins of the HSP60/10 family, molecular chaperonins, have been of great interest due to their ability to assist folding of many proteins both *in vivo* and *in vitro* (e.g. see Golubinoff *et al.*, 1989; van der Vies *et al.*, 1992). *Escherichia coli* GroEL is the best characterized chaperonin. It is a tetradecamer of identical subunits of 57 kDa each, organized in two stacked rings with 7-fold symmetry and a central cavity (Braig *et al.*, 1994). Partially folded proteins appear to bind inside the cavity in an ATP modulated manner. Under conditions where the spontaneous folding of substrate proteins does not occur, a co-chaperonin GroES is necessary for their release and productive folding (Weissman *et al.*, 1995). GroES is a heptamer of 10 kDa subunits that also exhibits a 7-fold symmetry (Hunt *et al.*, 1996). Each subunit is in a loose β-barrel like fold with two large extensions protruding out of it. One of the extensions is a β-hairpin that extends from the top of the barrel towards the protein symmetry axes. Together, the seven β-hairpins form a dome-like structure with an 8 Å orifice in the center (Hunt *et al.*, 1996). The other extension, the so-called mobile loop (residues 16 to 33) is on the bottom rim of the molecule and has been shown by NMR to be unstructured when GroES is free in solution (Landry *et al.*, 1993, 1996). This loop contains a significant number of hydrophobic residues and is highly conserved in evolution. It is responsible for the specific binding of GroES to GroEL during folding reaction. When bound to GroEL, the mobile loop assumes a β-hairpin conformation (Landry *et al.*, 1993, 1996). Upon binding, the smaller chaperonin forms a ''cap'' covering the central cavity of GroEL.

One important structural characteristic of GroES appears to be the high hydrophylicity of the inner surface of the dome, which contains a large number of exposed charged residues, particularly, the ring formed by Glu50 of each subunit that lines the oculus of the dome (Hunt *et al.*, 1996). Glutamates 53 form another ring around the orifice on the out-

Abbreviations used: CD, circular dichroism; DSC, differential scanning calorimetry.

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side of the dome. Interestingly, in cpn 10, an analog of GroES from *Mycobacterium leprae*, the cluster is extended to three carboxylic acid groups at positions 51, 52 and 53, and is followed by two more concentric rings of positively and negatively charged amino acid residues (Mande *et al.*, 1996). The high concentration of negatively charged residues at the top of the dome and the relatively weak packing interactions between β-hairpins have led to a proposition of metastability for this structure and the possibility that under physiological conditions it might be able to undergo a transition to a more open conformation (Hunt *et al.*, 1996).

Analysis of the crystal structure suggests that a number of hydrophobic interactions between the first β-strand of one subunit and the last β-strand of an adjacent subunit appear to play an important role in stabilizing the oligomeric structure (Hunt *et al.*, 1996). Consistently, truncation of seven amino acid residues on the carboxyl terminus results in the inability of the protein to form a heptamer (Seale & Horowitz, 1995). GroES has been shown to dissociate in urea with a transition midpoint around 3 M (Seale *et al.*, 1996). In sedimentation equilibrium experiments, heptameric GroES was found to exist in cooperative equilibrium with monomers with an equilibrium constant of 10^{38} M^{-6} or equivalently, an apparent ΔG of the order of −7.5 kcal per monomer (Zondlo *et al.*, 1995).

Here, the structural stability of GroES has been studied by means of differential scanning calorimetry (DSC) and circular dichroism (CD) under various solvent conditions. We show that the thermal folding/unfolding of GroES is a spontaneous reversible process involving a highly cooperative transition between folded heptamers and unfolded monomers. During the denaturation process, folded monomers are energetically unfavorable and consequently never become populated to an appreciable degree. The thermodynamic data also suggest that at room-temperature folded monomers are only marginally stable. Despite the high density of charged residues and ionic interactions, the stability of GroES shows no dependence on ionic strength at pH 7. On the other hand, millimolar concentrations of magnesium stabilize GroES considerably, suggesting a specific binding. Finally, a structural parameterization of the energetics developed previously (Bardi *et al.*, 1997; DAquino *et al.*, 1996; Gomez & Freire, 1995; Gomez *et al.*, 1995; Hilser *et al.*, 1996; Luque *et al.*, 1996) agrees well with the experimental thermodynamics of GroES and permits the identification of regions critical for stability.

Results and Discussion

Calorimetric experiments

The thermal stability of GroES was measured by high sensitivity differential scanning calorimetry. Figure 1 shows the partial molar heat capacity function of GroES at a concentration of 175 μM

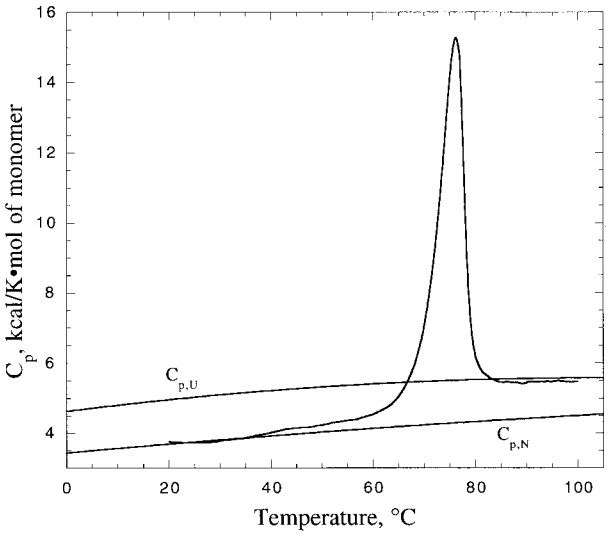

Figure 1. Partial molar heat capacity of GroES as function of temperature. GroES concentration was 175 μM of monomers. The continuous lines represent the heat capacities of the native, $C_{p,N}$, and unfolded, $C_{p,U}$, states, respectively, calculated as described (Gomez *et al.*, 1995).

(here, GroES concentrations are always expressed in terms of monomer units) in 20 mM sodium phosphate (pH 7.0). Under the conditions of this experiment, the thermal denaturation transition of GroES occurs at 76.4°C and is characterized by an enthalpy change (ΔH) of 63 kcal/mol and a change in heat capacity of 1.0 kcal K^{-1} mol^{-1}. At 25°C, the heat capacity of the native state is 3.7 kcal K^{-1} mol of $monomer^{-1}$ or 0.37 cal K^{-1} g^{-1}, which is slightly higher than the mean value of 0.34(±0.005) cal K^{-1} g^{-1} obtained for globular proteins (Gomez *et al.*, 1995). This is consistent with the NMR observation that 17 residues (16 to 32) are unstructured in native GroES when it is free in solution (Landry *et al.*, 1993, 1996), and with the absence of electron density for these residues in the crystal structure (Hunt *et al.*, 1996). Figure 1 also shows that the heat capacities of both native and unfolded GroES are in a good agreement with the values calculated using a structure based parameterization developed earlier (Gomez *et al.*, 1995). The similarity of the heat capacity of the denatured GroES and the heat capacity expected for an unstructured polypeptide of the same sequence indicates that the polypeptide chain is essentially unfolded and hydrated after thermal denaturation.

Figure 2 displays a set of DSC scans obtained at GroES concentrations of 36, 83 and 175 μM. In this Figure, the excess heat capacity functions obtained after subtracting the heat capacity of the native state are shown. It can be immediately noted that the transition temperature increases with protein concentration. This result indicates that the unfolding is coupled to the dissociation of the oligomeric protein. Also, the transition peaks are skewed towards the low temperature side of the transition

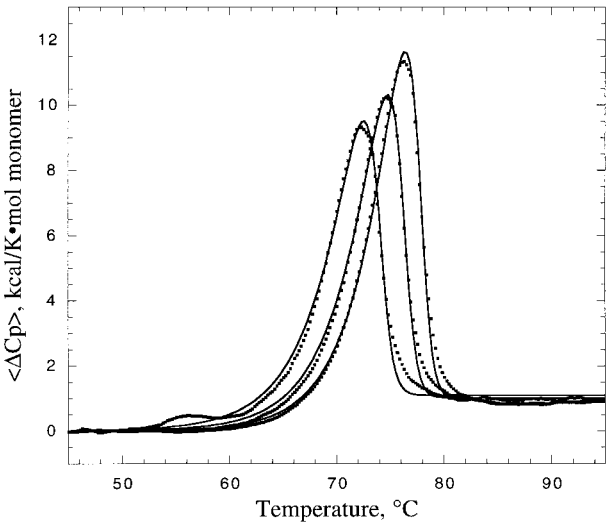

Figure 2. Excess heat capacity of GroES at different protein concentrations. GroES concentrations from left to right are 36, 87 and 175 μM. The continuous lines represent theoretical curves generated with the two-state folding/unfolding coupled to oligomerization model and the best set of fitting parameters obtained by non-linear least-squares optimization. The thermodynamic parameters corresponding to the fits are shown in Table 1.

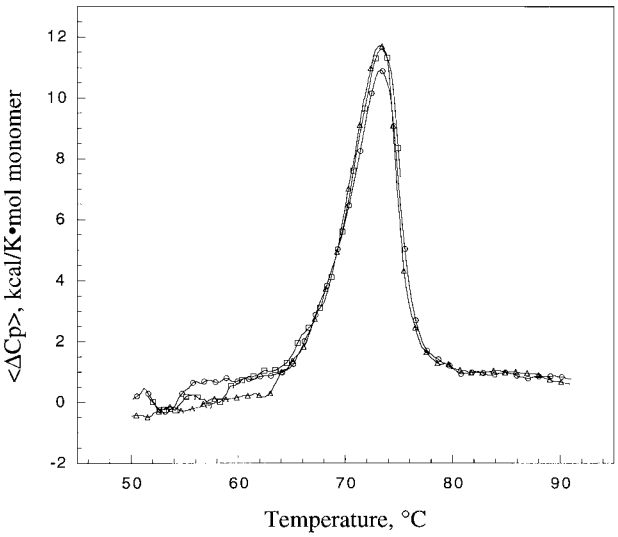

Figure 3. Test of the reversibility and scan rate independence of the thermal unfolding of GroES. Shown is the excess heat capacity of GroES obtained at 60 μM obtained at 1 deg. C per minute (open triangles) and 2 deg.C per minute (open circles and open squares). The difference between scans was within the experimental error (<0.1 deg.C). The curve labelled by open squares represents the first scan, which was allowed to proceed up to a temperature at which the transition was 83% completed. The curve labelled by open circles is the second scan of that sample.

as expected for a transition coupled to dissociation (Freire, 1989). Consequently, the temperature of the midpoint of the transition $T_{1/2}$ does not coincide with the temperature of the excess heat capacity maximum T_m. For the calorimetric scans shown in the Figure, the ratio of the van't Hoff and calorimetric enthalpies ($\Delta H_{VH}/\Delta H_{cal}$) averaged 1.83 ± 0.08. This value is significantly higher than 1.0, which is the expected value for the two-state unfolding of a monomeric protein. For the two-state unfolding of an oligomeric protein coupled to an association/dissociation process, the $\Delta H_{VH}/\Delta H_{cal}$ ratio can be expressed as (Freire, 1989):

$$\Delta H_{VH}/\Delta H_{cal} = 2n/(n+1) \qquad (1)$$

where n is the number of subunits and equals 1.75 for a heptamer, which is reasonably close to the experimentally obtained value.

The reversibility of the thermal denaturation of GroES was tested by performing repeated scans of the same samples. It was observed (Figure 3) that when the calorimetric scans were interrupted immediately after the transition, the unfolding reactions were fully reversible, indicating that the calorimetric experiments represented equilibrium conditions. However, prolonged incubation of the samples at high temperatures resulted in the appearance of an irreversible process. For example, incubation at 80°C or higher for longer than 10 to 15 minutes resulted in a significant broadening of the excess heat capacity peak and its shift to lower temperatures in repeated scans. Experiments performed at scanning rates of 1 and 2 deg. C per minute gave denaturation profiles differing by less than 0.1 deg. C (see also Figure 3), indicating the absence of kinetic effects under the conditions of these experiments.

The excess heat capacity functions obtained at different protein concentrations were analyzed by fitting the data to the two-state folding/unfolding oligomerization model (equation (8) in Materials and Methods) either individually or globally, using protein concentration as an additional variable. The highest likelihood values for ΔH, ΔS and ΔC_p that were obtained by the non-linear least-squares minimization procedure are shown in Table 1. The lines through the data points in Figure 2 were generated using the results of the individual fits. It can be noted that the calculated and experimental curves are in good agreement. Also, the parameters obtained from individual fits were similar to those obtained from the global fit, indicating that the two-state folding/unfolding oligomerization model accounts well for the overall thermodynamic parameters and cooperativity of GroES folding/unfolding, and for its concentration dependence. Attempts to include intermediate forms (i.e. folded monomers) in the fitting equations did not improve the goodness of the fit, indicating that the two-state model is sufficient to describe the system quantitatively and that folded monomers are not measurably populated during the transition. The slight deviation between calculated and experimen-

Table 1. Thermodynamic parameters of GroES unfolding

[GroES] (μM)	T_0 (°C)	T_m (°C)	$T_{1/2}$, (°C)	ΔC_P, (kcal/mol K)	$\Delta H(70)$, (kcal/mol)	$\Delta S(970)$, (kcal/mol K)	$\Delta G(70)$, (kcal/mol)	SD[a]
CD								
11.5	97.7	70.2	68.7	1.0[b]	74.0	196.5	6.6	0.02
15.0	105.0	71.6	69.6	1.0[b]	54.0	138.3	6.6	0.02
22.5	99.5	72.0	70.5	1.0[b]	68.0	179.2	6.5	0.02
30.0	99.6	73.4	71.9	1.0[b]	63.9	167.1	6.6	0.03
37.5	101.2	74.2	72.5	1.0[b]	63.9	167.1	6.6	0.03
45.0	100.2	74.5	73.0	1.0[b]	67.1	176.2	6.6	0.03
52.5	101.7	74.6	72.6	1.0[b]	60.1	156.5	6.4	0.03
Global fit (CD)	99.7	–	–	1.0[b]	68.0	179.0	6.6	0.003
DSC								
18.6	100.8	70.5	68.7	1.148	59.4	154.7	6.3	40.7
36.0	101.5	72.6	70.5	1.022	58.5	152.2	6.2	35.2
54.4	99.6	73.5	71.8	1.111	62.3	163.6	6.2	35.2
87.0	100.5	74.8	72.0	0.969	61.2	160.4	6.1	13.6
148.0	98.9	75.9	74.2	1.096	63.5	167.2	6.1	22.2
175.0	99.4	76.4	74.7	0.998	63.0	165.9	6.1	15.6
Global fit (DSC)	99.5	–	–	1.029	63.6	167.4	6.2	18.6

[a] Standard deviation.
[b] To fit CD data, the value of ΔC_P was fixed.

tal curves observed at the high temperature end of the transition could be due to the onset of irreversible processes.

CD experiments

Circular dichroism (CD) experiments were utilized to study the thermal transition of GroES in the lower range of protein concentrations. Figure 4 displays a series of wavelength scans from 240 to 190 nm at increasing temperatures. GroES is a predominantly β-sheet protein and thus its negative molar ellipticity is relatively low. Upon heating a sample to temperatures above transition, the shape of the spectrum changes significantly and becomes characteristic of a random coil. The thermal denaturation of GroES was monitored by following the changes in molar ellipticity at 222 nm, since at this wavelength the ellipticity decreases significantly upon unfolding of the protein and both the pre and post-transitional baselines show little temperature dependence. Figure 5 shows a series of temperature scans obtained for a range of GroES monomer concentrations between 11 and 55 μM. As in the case of the DSC experiments, the midpoint of the transition increases with the increase of protein concentration and the curves are skewed towards the low temperature side. The data were

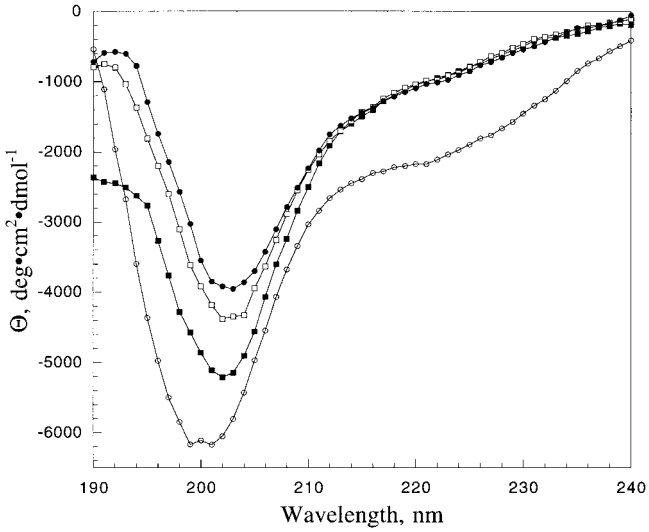

Figure 4. Representative CD spectra of GroES at different temperatures. Temperature was increased from 10 to 95°C at a rate of 1 deg.C per minute and the spectra of the protein were recorded at 12°C (■), 37°C (□), 60°C (●) and 80°C (○).

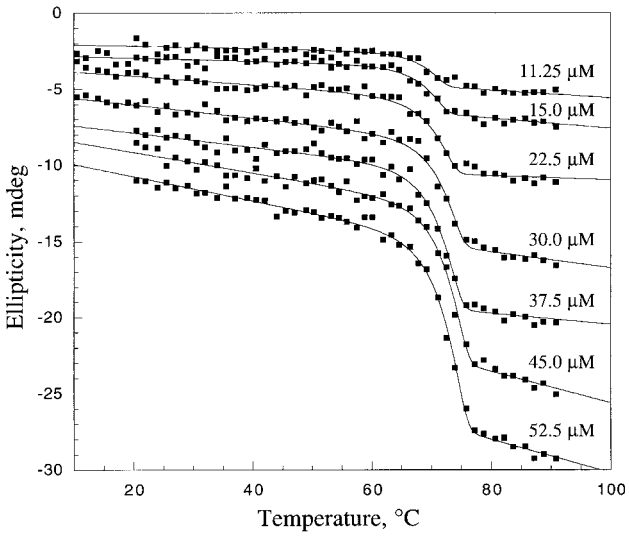

Figure 5. Elipticity of GroES solutions at 222 nm as a function of temperature. Protein concentrations are, from top to bottom: 11.25, 15.0, 22.5, 30.0, 37.5, 45.0, 52.5 μM, respectively. The continuous lines represent theoretical curves resulting from the fitting of the individual data sets. The thermodynamic parameters corresponding to the curves are shown in Table 1.

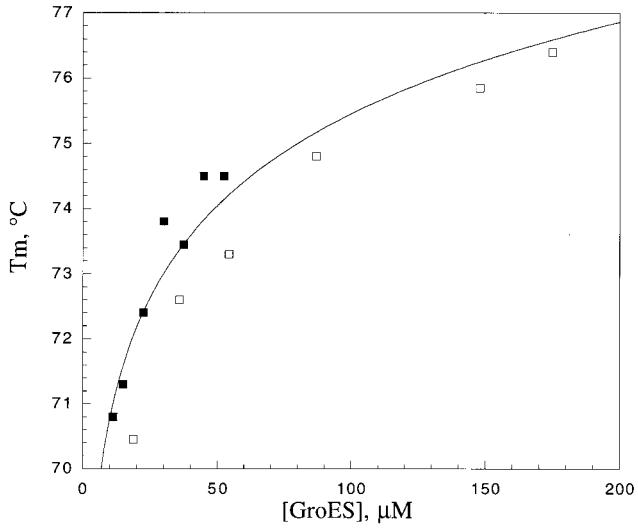

Figure 6. Unfolding transition temperature, T_m, of GroES as a function of protein concentration. Open squares represent temperatures of the excess heat capacity maxima obtained from DSC experiments. Filled squares represent temperatures at which the rate of change of GroES ellipticity at 222 nm with temperature is maximum. The continuous line is a logarithmic fit through the data points. The difference of 1 deg.C in the absolute temperature values reported by the CD and DSC is due to differences in the temperature calibrations of the instruments.

analyzed as described above assuming the simplest model of a two-state protein unfolding coupled to oligomer dissociation. The curves were fitted using non-linear least-squares minimization either for individual data sets (lines through the data points in Figure 5) or globally. When a global minimization procedure was applied to fit the temperature dependence of the molar ellipticity, the protein concentration was used as an additional variable. In both types of analysis, only ΔH and ΔS were optimized. ΔC_p is not accessible directly from CD experiments, and thus it was fixed to 1.0 kcal K^{-1} mol^{-1}.

The thermodynamic parameters obtained from the analysis of the CD experiments are also summarized in Table 1. As in the case of the DSC data, the thermodynamic parameters obtained from the global fitting procedure are very close to the values obtained from the fitting of individual curves, indicating that the two-state folding/unfolding oligomerization model provides a good approximation to the data. The magnitude of the resulting thermodynamic parameters are similar to those obtained from the DSC data indicating that the two techniques measure the same process over the entire concentration range studied. Together, DSC data and CD experiments cover a protein concentration range of 11.5 to 175 μM. In Figure 6 the temperatures of the midpoint of the transition obtained by both techniques are plotted *versus* protein concentration.

Urea dependence of unfolding and GroES stability at 25°C

The urea dependence of the stability of GroES was assessed by performing DSC experiments in the presence of 0 to 3 M urea (data not shown). Although the stability of the protein dropped significantly (at 3 M urea and a protein concentration of 31 μM, the transition midpoint was 48.5°C compared to 72.5°C in the absence of urea), the transition remained cooperative and fitted well to the two-state folding/unfolding oligomerization model. From the temperature dependence of the enthalpy values obtained in the presence of urea, ΔC_p was estimated to be around 1.37 kcal K^{-1} mol^{-1}, which is somewhat higher than that obtained in the absence of urea (see Table 1). Slightly higher ΔC_p values in the presence of urea have been reported previously for other proteins (Makhatadze & Privalov, 1992).

The calculated Gibbs energy of stabilization of GroES at 25°C, $\Delta G(25)$, ranges between 9.0 and 10.2 kcal/mol according to the DSC thermal denaturation data obtained both in the absence and in the presence of urea. The urea unfolding of GroES at 25°C has been reported by Seale *et al.* (1996) and is reproduced in Figure 7. The data were fit to the same two-state folding/unfolding oligomerization model used to fit the DSC and CD data. In this case, the dependence of ΔG on urea concentration was represented by the standard linear extrapolation equation:

$$\Delta G = \Delta G_0 - m\,[\text{Urea}] \qquad (2)$$

where ΔG_0 is the free energy change in the absence of urea. The data were analyzed by non-linear

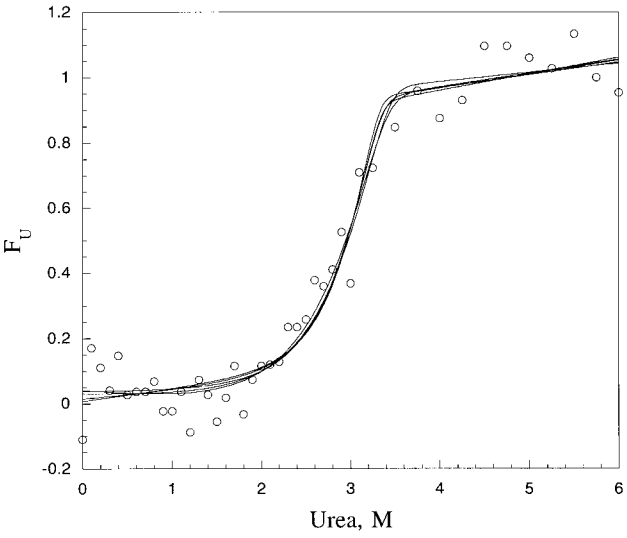

Figure 7. Fraction of GroES molecules in the unfolded state as a function of urea concentration. The data points are from Seale *et al.* (1996) and were obtained at 25°C. The continuous lines are the best-fit curves using the two-state folding/unfolding coupled to oligomerization model (see the text for details).

least-squares minimization. Pre and post-transitional baselines were considered linear, as in the case of the CD data. ΔG_0 values ranging between 8 and 10 kcal/mol with corresponding m values ranging between 0.98 and 1.64 kcal l/mol^2 were all consistent with the experimental data, yielding curves that could not be discriminated (Figure 6). These ΔG_0 values overlap with those derived from temperature denaturation experiments, suggesting that the same equilibrium might be observed in both cases. However, a wider range of ΔG_0 and m values fit the data equally well. The Gibbs energies derived from either thermal or urea denaturation experiments are also close to the value of 7.5 kcal/mol obtained from sedimentation equilibrium for the heptamer-monomer equilibrium (Zondlo *et al.*, 1995). The ΔG values obtained by measurements at 25°C are similar to those extrapolated from thermal denaturation experiments in which the population of the folded monomers during the transition is negligible. The difference of at most 1.5 kcal is within the extrapolation error. Nevertheless, even if it is assumed to be real, all the available data indicate that isolated monomers are unstable or, at most, only marginally stable at 25°C.

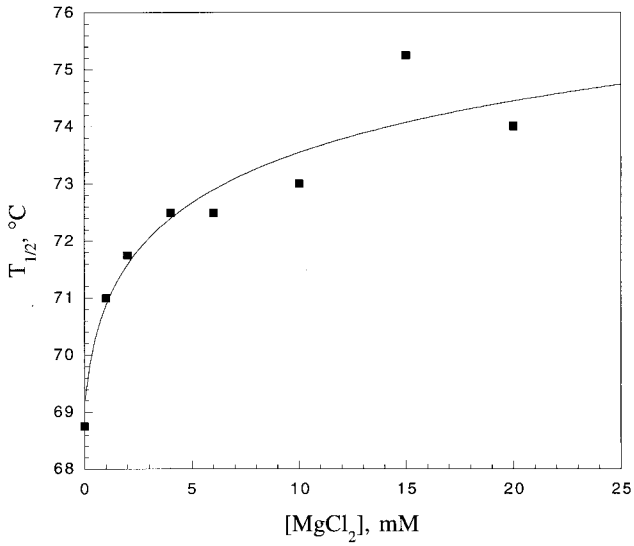

Figure 8. Midpoint temperature of the unfolding transition, $T_{1/2}$ of GroES as a function of $MgCl_2$ concentration. The continuous line through the data points was generated using the highest likelihood parameters obtained from the global fit of the data.

Effects of magnesium ions on the stability of GroES

Thermal denaturation experiments were performed in the presence of varying concentrations of KCl. Salt concentrations as high as 1 M had no detectable effect on the T_m of GroES unfolding. In contrast, even low concentrations of $MgCl_2$ stabilized the protein considerably (see Figure 8), suggesting that GroES binds Mg^{2+} stoichiometrically. If this is the case, the Gibbs energy for unfolding in the presence of Mg^{2+} can be written as:

$$\Delta G' = \Delta G + NRT \ln\left(1 + \frac{1}{K_D}[Mg^{2+}]\right) \qquad (3)$$

assuming that the unfolded state does not bind Mg^{2+} and that the native state has N identical and independent sites. Since the concentrations of Mg^{2+} at which the effects are seen are much higher than the protein concentration (15 μM) used in the experiments, the free Mg^{2+} concentration is well approximated by the total Mg^{2+} concentration present in the solution. The above equation was introduced into the two-state folding/unfolding oligomerization model in order to analyze a set of temperature-induced unfolding experiments performed at different concentrations of $MgCl_2$ and evaluate the binding dissociation constant K_D and the number of binding sites N. For simplicity, K_D was assumed to be constant in the small temperature range in which GroES unfolding occurs. A global analysis of the CD data was carried out as described above except that the protein concentration was kept constant and the $MgCl_2$ concentration was used as an additional variable. The thermodynamic parameters corresponding to the free protein (ΔH, T_0 and ΔC_p) were kept constant and set to the best-fit parameters obtained for the 0 M $MgCl_2$ experimental point. ΔC_p was fixed to 1.0 kcal/(mol K) as described above and ΔH and T_0 were set to 62 kcal/mol and 101.4°C, respectively. Only N and K_D were varied during the non-linear least-squares minimization. The highest likelihood value for K_D was found to be 0.43(±0.36) mM or in terms of Gibbs free energy 5.3(±0.7) kcal/mol at the temperature of transition. The number of binding sites was 0.37(±0.09) per monomer of GroES or around 2.6 per heptamer. The errors in the values correspond to 95% confidence interval for the best fit parameters. The continuous line in Figure 8 was generated using those values and describes the dependence of T_m on $MgCl_2$ concentration reasonably well.

The existence of metal-binding sites in GroES is consistent with the diffuse binding of heavy metals observed during crystallization (Hunt *et al.*, 1996). This binding was observed inside an 8 Å orifice at the top of the dome-like structure formed by the seven β-hairpin extensions that include residues 46 through 57 of each subunit. In particular, Glu50 and Glu53 define two rings of negatively charged amino acid residues close to the top of the dome and are the most likely candidates to contribute to the ligation of divalent cations. The stoichiometry derived from the denaturation experiments also suggests that the Mg^{2+}-binding sites are defined by more than one subunit.

Structural thermodynamic calculations

The crystal structure of GroES has been solved to 2.8 Å resolution (Hunt *et al.*, 1996) and was used to perform structure-based thermodynamic calculations as described (Bardi *et al.*, 1997; DAquino *et al.*, 1996; Gomez & Freire, 1995; Gomez *et al.*, 1995; Hilser *et al.*, 1996; Luque *et al.*, 1996).

In the structural parameterization the Gibbs energy, ΔG, is decomposed into a generic, ionic and translational components. The generic portion of the Gibbs energy, ΔG_{gen}, is calculated from a separate computation of its enthalpy and entropy components. This portion of the Gibbs energy contains those contributions typically associated with the formation of secondary and tertiary structure (van der Waals interactions, hydrogen bonding, hydration and conformational entropy). The remaining contributions to the Gibbs energy of folding are not separated into enthalpic and entropic components. They include excess electrostatic and ionization effects, ΔG_{ion}, and the contribution of the change in translational degrees of freedom, ΔG_{tr}:

$$\Delta G = \Delta G_{gen} + \Delta G_{ion} + \Delta G_{tr} \quad (4)$$

In GroES, residues 16 through 33 are located in a mobile loop that is believed to be largely unfolded in uncomplexed GroES (Landry *et al.*, 1993, 1996). In the crystallographic structure, the amino acid residues in this region are resolved in only one of the seven subunits (subunit B) and unresolved in the remainder. It has been suggested that the observed structure for this loop results from the packing between two molecules of GroES in the crystal (Hunt *et al.*, 1996). Two different cases were considered in the structure-based thermodynamic calculations. In one case, residues 16 through 33 were assumed to be in the conformation observed in subunit B; and, in the second case they were assumed to be unstructured. Table 2 summarizes the calculated enthalpy and heat capacity values obtained for the complete unfolding of heptameric GroES. It is clear from the Table that the agreement between calculated and experimental results is better for the case in which residues 16 through 33 are assumed to be unstructured. In particular, the enthalpy change predicted in the case in which residues 16 through 33 are structured is much higher than the experimental value, while the enthalpy change predicted when these residues are unstructured closely matches the experimental value. In the remaining calculations, case 2 will be considered.

Table 2. Enthalpy and heat capacity changes for unfolding of heptameric GroES

	GroES case 1	GroES case 2	GroES experimental
ΔC_p (cal/K mol)	1413	1273	1029[a] 1370[b]
$\Delta H(70)$ (cal/mol)	77,831	62,648	63,600[a]

In case 1, residues 16 through 33 are assumed to be structured. In case 2, residues 16 through 33 are assumed to be unstructured. See the text for details.

[a] Values obtained in the global fitting of DSC data.

[b] Value obtained from urea experiments.

For discussion purposes, the formation of the folded GroES heptamer can be considered as being composed of two steps: the folding of isolated monomers into a conformation identical with that existing in the heptamer and their association to form the heptameric structure. This type of dissection allows evaluation of the Gibbs energy for each step. The calculated thermodynamic parameters for those two processes are summarized in Table 3. As indicated in the Table, the folding of an isolated monomer into the conformation existing in the heptamer is very unfavorable and expected to be characterized by a Gibbs energy of unfolding of the order of −11 kcal/mol at 25°C. This value suggests that if isolated folded monomers do exist they must have a different structure from that observed in the heptamer. On the average, intersubunit interactions should contribute close to 20 kcal/mol to bring the overall Gibbs energy to the experimentally observed value (Table 3).

As shown in Table 3, individual GroES monomers form a substantial hydrophobic core and gain close to 80% of the total solvent entropy, ΔS_{solv} arising from the release of water molecules upon burial of hydrophobic surfaces. However, the isolated monomers are unstable because they do not form a compact structure in which a large fraction of the amino acid residues becomes buried from the solvent and establish significant van der Waals and hydrogen bonding interactions. Only 52% of the non-polar surface and 44% of the polar surface are buried from the solvent in a GroES isolated monomer as compared to 61% and 57%, respectively, typical for globular proteins with a similar number of residues. Consequently, upon folding, GroES monomers gain only 20% of the total favorable enthalpy. In contrast, they loose 92% of the overall conformational entropy, ΔS_{conf} as they form a rigid structure. Interestingly, electrostatic interactions between charged groups make a favorable contribution to monomer stability.

The stabilization of the GroES heptamer is due to the interactions existing between subunits. The magnitude of these interactions overcome the unfavorable Gibbs energy of folding a monomer. As shown in Table 3, the intersubunit interactions are energetically favorable both enthalpically and entropically. The favorable enthalpy is due to the formation of a compact structure (67% non-polar and 58% polar surface buried from the solvent) and the favorable entropy is due to the gain in solvent entropy upon burial of the remaining hydrophobic surface and a very small loss of conformational entropy upon interaction of already folded subunits.

Table 3. Dissection of folding energetics for GroES (298.15 K)

Parameter	Folding	Oligomerization	Total
ΔASA_{ap} (Å^2)	3414	2004	5418
ΔASA_{pol} (å^2)	2074	1407	3481
ΔC_p (cal/K mol)	1008	265	1273
ΔH_{gen} (cal/mol)	1023	4340	5363
ΔS_{conf} (cal/K mol)	386	34	420
ΔS_{solv} (cal/K mol)	−329	−93	−422
ΔG_{gen} (cal/mol)	−16,001	21,952	5951
ΔG_{ion} (cal/mol)	4363	−1970	2393
ΔG_{tr} (cal/mol)	0	−886	−886
ΔG_{total} (cal/mol)	−11,640	19,100	7458

The native state is the reference state. Thermodynamic values are per mol of monomer.

Structure-based thermodynamic parameters as defined in references: Bardi *et al.* (1997); DAquino *et al.* (1996); Gomez & Freire (1995); Gomez *et al.* (1995); Hilser *et al.* (1996); Luque *et al.* (1996). ΔASA_{ap} and ΔASA_{pol} are the changes in apolar and polar solvent accessibilities. ΔC_p is the change in heat capacity, ΔH_{gen} and ΔG_{gen} are the generic changes in enthalpy and free energy. ΔS_{conf} and ΔS_{solv} are the conformational and solvation entropy changes. ΔG_{ion} and ΔG_{tr} are the electrostatic and translational changes in free energy. ΔG_{total} is the total change in free energy.

Overall, electrostatic interactions contribute unfavorably to the association reaction. Although the electrostatic component of the Gibbs free energy cannot be partitioned into contributions per residue, it is likely that the two rings of negative charge (particularly Glu50 and Glu53) that line the oculus of the GroES dome are the major determinants of these unfavorable interactions as has been suggested by Hunt *et al.* (1996). Overall, however, the results suggest that the remaining interactions are sufficient to compensate the repulsive electrostatic component and still favor the closed structure observed in the crystal. Residues 47 through 56, which comprise the major part of the dome, contribute around 2.6 kcal to the stability of the heptamer. The structure is favorable both enthalpically due to sufficiently tight packing to form van der Waals contacts and hydrogen bonds, and entropically, since it buries a significant hydrophobic surface.

As can be expected for a protein with a large fraction (33%) of charged residues, electrostatic interactions play an important role in determining the stability of the protein. However, it is noteworthy that favorable contributions to the folding of monomers are at least partially compensated by the unfavorable electrostatic effects on oligomerization. It is also interesting that even though overall electrostatic interactions appear to be favorable, no salt destabilization of GroES has been observed. These results parallel the evidence obtained for other proteins, which seem to indicate that electrostatic interactions between carboxylic groups show little salt dependence (Swint-Kruse & Robertson, 1995).

Conclusions

The thermal unfolding of GroES has been found to be reversible and to obey a simple two-state folding/unfolding oligomerization reaction. In this mechanism, unfolded monomers exist in equilibrium with fully folded heptamers with no measurable population of intermediate species. When extrapolated to 25°C, the Gibbs energy of stabilization of GroES is around 8 to 10 kcal/mol per monomer.

Structure-based thermodynamic calculations of the thermodynamic parameters of GroES unfolding yield values for ΔG, ΔH and ΔC_p that are in general agreement with the experimental values, provided that the loop defined by residues 16 through 33 is assumed to be unstructured as suggested by the crystallographic and NMR results. The lack of stability of isolated monomers appears to be due to the absence of a closely packed structure in which residues establish close van der Waals contacts and bury a significant surface from the solvent. The structural analysis suggests that electrostatic interactions contribute favorably to folding but unfavorably to oligomerization. As reported earlier for p53 (Johnson *et al.*, 1995) and the leucine zipper of GCN4 (Kenar *et al.*, 1995; Thompson *et al.*, 1993), the results obtained with GroES emphasize the dominating role of quaternary interactions in the stabilization of small oligomeric proteins.

Materials and Methods

Protein purification

Expression of GroES was carried out in *Escherichia coli* BL-21 kanamycin resistant cells transformed with pGroESL plasmid, which provided also a chloramphenicol resistance. Protein was overexpressed and purified using a modification of a previously published procedure (Todd *et al.*, 1993). Briefly, 2 to 4 l of cells were grown at 37°C to an absorbance of 0.8 at 600 nm and induced by 1 mM of isopropyl thiogalactoside for two hours. Cells were harvested by centrifugation, resuspended in buffer (20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 10 mM DTT, 0.1 mM PMSF) and lysed using a French press by applying a pressure of 20,000 psi. Cell lysate was clarified by centrifugation and the supernatant was applied to a DEAE-Sepharose CL-4B (Pharmacia Inc.) FPLC column equilibrated with 20 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM DTT. GroES was eluted with a 0 to 1 M gradient of potassium phosphate and was detected by SDS-PAGE. Fractions containing GroES were precipitated with saturated ammonium sulfate (30 minutes at 4°C), collected by centrifugation, re-suspended in 20 mM Tris-HCl (pH 7.8), 1.2 M ammonium sulfate, 0.5 mM EDTA

and applied onto a Phenyl Sepharose (Pharmacia Inc.) column equilibrated in the same buffer. GroES was eluted with a 1.2 M to 0.5 M gradient of ammonium sulfate and detected by SDS-PAGE. GroES-containing fractions were concentrated using Amicon ultrafiltration cells (Amicon Inc.) with a 3 kDa molecular mass cutoff Diaflo ultrafiltration membrane (Amicon Inc.). GroES was diluted tenfold into 40 mM succinate (pH 4.5) and applied to a MonoS (Pharmacia Inc.) column. The cation-exchange column was developed with a 0 to 1 M NaCl gradient and the purity was assessed by gel electrophoresis. GroES appeared on SDS-PAGE as a single band with no detectable contamination. Protein was stored at 4°C in 20 mM sodium phosphate (pH 7.0) and 0.05% (w/v) of sodium azide.

Prior to each experiment, protein was dialyzed overnight against 4 l of the appropriate buffer using Spectra/Por 3500 kDa molecular mass cutoff dialysis membrane. Concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Differential scanning calorimetry (DSC)

Various concentrations of GroES in 20 mM sodium phosphate (pH 7.0), were scanned at 1 deg. C per minute using a high-precision differential scanning calorimeter N DSC (Calorimetry Science Corp.). Sample and reference solutions were properly degassed and carefully loaded into the cells to avoid bubble formation. Exhaustive cleaning of the cells was undertaken before each experiment. Data were analyzed by non-linear least-squares fitting programs developed in this laboratory. Excess heat capacity curves were analyzed both individually and globally using protein concentration as an additional variable.

Two different models were considered in the analysis. The first corresponds to a two-state folding/unfolding reaction coupled to oligomerization. In the second model this reaction is considered to include intermediates (i.e. folded monomers). The first case is the simplest model and corresponds to the situation in which the oligomeric protein obeys the following equilibrium:

$$\mathrm{M}_n \leftrightarrows n\mathrm{U} \tag{5}$$

where M_n and U represent a folded oligomer and unfolded monomer, respectively. Theoretical treatment of such an equilibrium has been developed (Freire, 1989; Johnson & Freire, 1996; Johnson *et al.*, 1995; Thompson *et al.*, 1993) and applied to dimeric and tetrameric systems. At all temperatures, the fraction of protein in the unfolded state, F_U can be obtained from the equation:

$$F_\mathrm{U} = \frac{[\mathrm{U}]}{[\mathrm{U}] + n\mathrm{M}_n} = \frac{K^{1/n}[\mathrm{M}_n]^{1/n}}{K^{1/n}[\mathrm{M}_n]^{1/n} + n[\mathrm{M}_n]} \tag{6}$$

where K is the equilibrium constant in equation (5). After rewriting this equation in terms of the total protein concentration $[\mathrm{P_T}]$, an expression for F_U can be derived:

$$\frac{F_\mathrm{u}}{(1-F_\mathrm{U})^{1/n}} = \frac{K^{1/n}[\mathrm{P_T}]^{1/n-1}}{n^{1/n}} \tag{7}$$

Equation (3) is a transcendental equation and needs to be solved numerically for F_u for any given values of the remaining parameters. The differential scanning calorimeter measures the excess heat capacity function, $\langle \Delta C_\mathrm{p} \rangle$, which is simply the temperature derivative of the average excess enthalpy, $\langle \Delta H \rangle$:

$$\begin{aligned}\langle \Delta C_\mathrm{p} \rangle &= \frac{\partial \langle \Delta H \rangle}{\partial T} = \frac{\partial F_\mathrm{U}}{\partial T}\Delta H + F_\mathrm{U}\Delta C_\mathrm{p} \\ &= \frac{\mathrm{n}F_\mathrm{U}(1-F_\mathrm{U})}{n(1-F_\mathrm{U})+F_\mathrm{U}}\frac{\Delta H^2}{RT^2} + F_\mathrm{U}\Delta C_\mathrm{p}\end{aligned} \tag{8}$$

where ΔH and ΔC_p are changes in the enthalpy and heat capacity upon unfolding, respectively.

The equilibrium constant K can be written in terms of the free energy change ΔG, $K = \exp(-\Delta G/RT)$ where ΔG is:

$$\begin{aligned}\Delta G = \Delta H(T_0) + \Delta C_p(T - T_o) \\ - T\left(\Delta S(T_0) + \Delta C_\mathrm{p}\ln\left(\frac{T}{T_0}\right)\right)\end{aligned} \tag{9}$$

For convenience, the reference temperature T_0 was chosen as the temperature at which $\Delta G(T_0) = 0$. It must be noted that for the unfolding of an oligomeric protein T_0 does not coincide with the temperature of the midpoint of the transition or the maximum in the excess heat capacity function. The above equations were used to fit the data and obtain the best estimates for the three parameters ΔH, ΔS and ΔC_p. To avoid local minima in multiple parameter space one of the parameters, usually ΔH, was first varied systematically over a range of values. For every value, the best fit was determined and the set of parameters that produced the smallest sum of squared residuals was selected. This procedure was further improved by optimizing all the parameters simultaneously in order to obtain the highest likelihood values for all the parameters.

Circular dichroism spectroscopy (CD)

CD experiments were conducted using a Jasco J-710 spectropolarimeter with a Peltier type cell-holder (model PTC-348W from Jasco Corporation), which permits accurate temperature control. Wavelength scans were performed using 36 μM GroES (concentration is expressed in terms of monomers) in a 1 mm rectangular cell at a number of discrete temperatures. Each spectrum was obtained by averaging ten spectra recorded from 240 to 190 nm with 1 nm intervals at the rate of 50 nm per minute. A response time for each point was eight seconds and the band width was 1 nm. Buffer scans were accumulated and subtracted from the sample scans, and the mean residue ellipticity was calculated.

CD temperature scans were performed by varying the temperature from 10 to 95°C at a rate of 1 deg.C per minute and the mean ellipticity was measured at 222 nm with 0.5° intervals, eight seconds response time and 1 nm bandwidth. GroES melting curves were analyzed using a non-linear least-squares fitting program. Individual curves were fit to the equation:

$$\theta = (1 - F_\mathrm{U})(a_1 + a_2T) + F_\mathrm{U}(b_1 + b_2T) \tag{10}$$

where θ is the ellipticity at any temperature and a_1 and a_2, b_1 and b_2 describe pre and post-transitional linear baselines, respectively. The population of the unfolded state F_U at any temperature is expressed as a function of the thermodynamic parameters as described above. When using the global analysis for a set of unfolding experiments, the fraction of unfolded heptamer F_U was

calculated from the ellipticity prior to the minimization procedure according to the relationship:

$$F_U = (\theta - \theta_N)/(\theta_U - \theta_N) \quad (11)$$

$\theta_N = a_1 + a_2T$ and $\theta_U = b_1 + b_2T$ are mean ellipticities of folded and unfolded states, respectively, and were obtained by linear regressions of pre and post-transitional baselines. To avoid local minima in parameter space, the data were analyzed in a manner similar to the analysis of DSC data as described above.

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