FOR THE RECORD

Mechanical unfolding of covalently linked GroES: Evidence of structural subunit intermediates

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Abstract: It is difficult to determine the structural stability of the individual subunits or protomers of many proteins in the cell that exist in an oligomeric or complexed state. In this study, we used single-molecule force spectroscopy on seven subunits of covalently linked cochaperonin GroES (ESC7) to evaluate the structural stability of the subunit. A modified form of ESC7 was immobilized on a mica surface. The force-extension profile obtained from the mechanical unfolding of this ESC7 showed a distinctive sawtooth pattern that is typical for multimodular proteins. When analyzed according to the worm-like chain model, the contour lengths calculated from the peaks in the profile suggested that linked-GroES subunits unfold in distinct steps after the oligomeric ring structure of ESC7 is disrupted. The evidence that structured subunits of ESC7 withstand external force to some extent even after the perturbation of the oligomeric ring structure suggests that a stable monomeric intermediate is an important component of the equilibrium unfolding reaction of GroES.

Keywords: force spectroscopy; mechanical unfolding intermediates; protein subunit stability; covalently linked oligomeric protein; GroES; cpn10

Abbreviations: AFM, atomic force microscopy; CH-ESC7, ESC7 that has two-cysteine residues and six-histidine residues extending from the N terminus and C terminus, respectively; ESC7, engineered form of cochaperonin GroES with covalently linked subunits; Gdn-HCl, guanidine hydrochloride; PBS, phosphate saline buffer.

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Introduction

Many cellular proteins are multisubunit or multidomain proteins with complex structures; these proteins are capable of sustaining complex functions involving numerous regulatory aspects. The relative abundance and importance of these proteins make them a natural subject of studies regarding protein folding and structural stability. Unfortunately, most of these studies often encounter difficulties, because these proteins tend to misfold or misassemble and form aggregates during unfolding and refolding experiments. Even when the protein shows a reversible folding reaction that makes detailed analysis possible, the folding and unfolding processes tend to involve numerous intermediates, which complicate interpretation of the experimental data.

With regard to oligomeric proteins, most folding studies have been performed with low-order oligomeric proteins such as dimers,[1] whereas studies with higher-order oligomeric proteins have been relatively rare. Recently, GroES, a heptameric homo-oligomer from *E. coli*, was found to fold essentially reversibly in *in vitro* unfolding and refolding experiments.[2–4] GroES consists of seven β-barreled 10 kDa subunits that form a ring-like quaternary structure.[5,6] The full reversibility of GroES folding was examined at protein concentrations as high as 12 mg mL^{-1}.[7] Therefore, GroES provides an ideal model system for the study of the folding and structural stability of multisubunit proteins.

Analysis of the equilibrium unfolding/refolding of GroES using guanidine hydrochloride (Gdn-HCl) as a denaturant revealed that unfolding of the protein is expressed by a three-state model; in the second state, the GroES heptamer is dissociated into marginally stable monomers, and in the third state, these monomers are unfolded completely.[4] The major contribution to the overall stability of GroES was from intersubunit interactions, but our results showed that a minor contribution from subunit folding was also important. Folding studies of cpn10 from other species (human, rat, *T. maritima*, and *A. aeolicus*) have been performed, and it was confirmed that the folding was reversible.[8–10] However, there are discrepancies in the unfolding models among studies using different proteins and denaturants. Guidry[8] and Luke[10] have reported that equilibrium unfolding of human cpn10 protein can be expressed by a two-state model and that a denatured heptameric state may be seen in experiments using urea. These differences may indicate that the folding and unfolding pathways of these proteins are determined by a delicate balance among free energy levels of numerous states.

Recent advances in atomic force microscopy (AFM) technology have made it possible to manipulate single protein molecules. The new experimental techniques have been employed in protein folding studies where mechanical force was used as a denaturation method.[11–13] Here, to examine whether the monomeric unfolding intermediate of GroES actually exists during unfolding, we applied this mechanical unfolding method to GroES. A GroES protein whose subunits had been covalently linked was immobilized on mica and extended (unfolded) mechanically in solution using AFM. The force-extension profile obtained from the experiments showed a distinctive sawtooth pattern, suggesting that individual subunits unfold stochastically after the disruption of the GroES ring structure and that a dissociated GroES monomer may exist in stable form during unfolding.

Results and Discussion

In a previous work, we studied the structural stability of a single-polypeptide-chain variant of GroES (ESC7), which was constructed by consecutively linking the C terminus of one subunit to the N terminus of an adjacent subunit with a three-glycine linker peptide.[14] We found that ESC7 displays two-state cooperative transitions in equilibrium unfolding experiments but shows multiphasic kinetic profiles, which indicated the existence of transient intermediates.

For this study, we constructed a modified variant of ESC7 (CH-ESC7) for mechanical manipulation [Fig. 1(A)]. CH-ESC7 has two-cysteine residues and six-histidine residues extending from the N terminus and C terminus, respectively. Purified CH-ESC7 showed activities similar to wild-type GroES (Table I), indicative of the fact that CH-ESC7 possesses a native-like fold. CH-ESC7 was immobilized on a mica surface that had been premodified with mercapto groups, through disulfide bond formation with the N-terminal cysteine residue. The AFM image of CH-ESC7 immobilized on the treated mica surface [Fig. 1(B)] shows a discrete distribution of CH-ESC7. The distance between neighboring CH-ESC7 molecules was greater than 20 nm, which was sufficient to allow a single CH-ESC7 molecule to be selected with the AFM gold-coated probes used in this study.

In PBS solution, the immobilized CH-ESC7 molecules were picked up and extended by the probe. We had initially intended for the protein to adhere to the probe through interactions between the six histidine residues at the C-terminal and the probe itself.[15] However, we could not rule out the possibility of nonspecific interactions. A typical force-extension profile is shown in Figure 1(C). The profile shows a distinctive sawtooth pattern that is typical for mechanical unfolding profiles of multimodule proteins, such as titin and tenascin.[12,16,17] We found eight significant peaks [peaks **1**–**8** in Fig. 1(C)], not counting the peak caused by nonspecific interaction at 0 nm distance. The gradual ascent of each peak is directly related to an extension of the unstructured polypeptide chain, whereas the steep descent of each peak is directly related to the recoiling of the cantilever resulting from a sudden increase in the length of the unstructured polypeptide

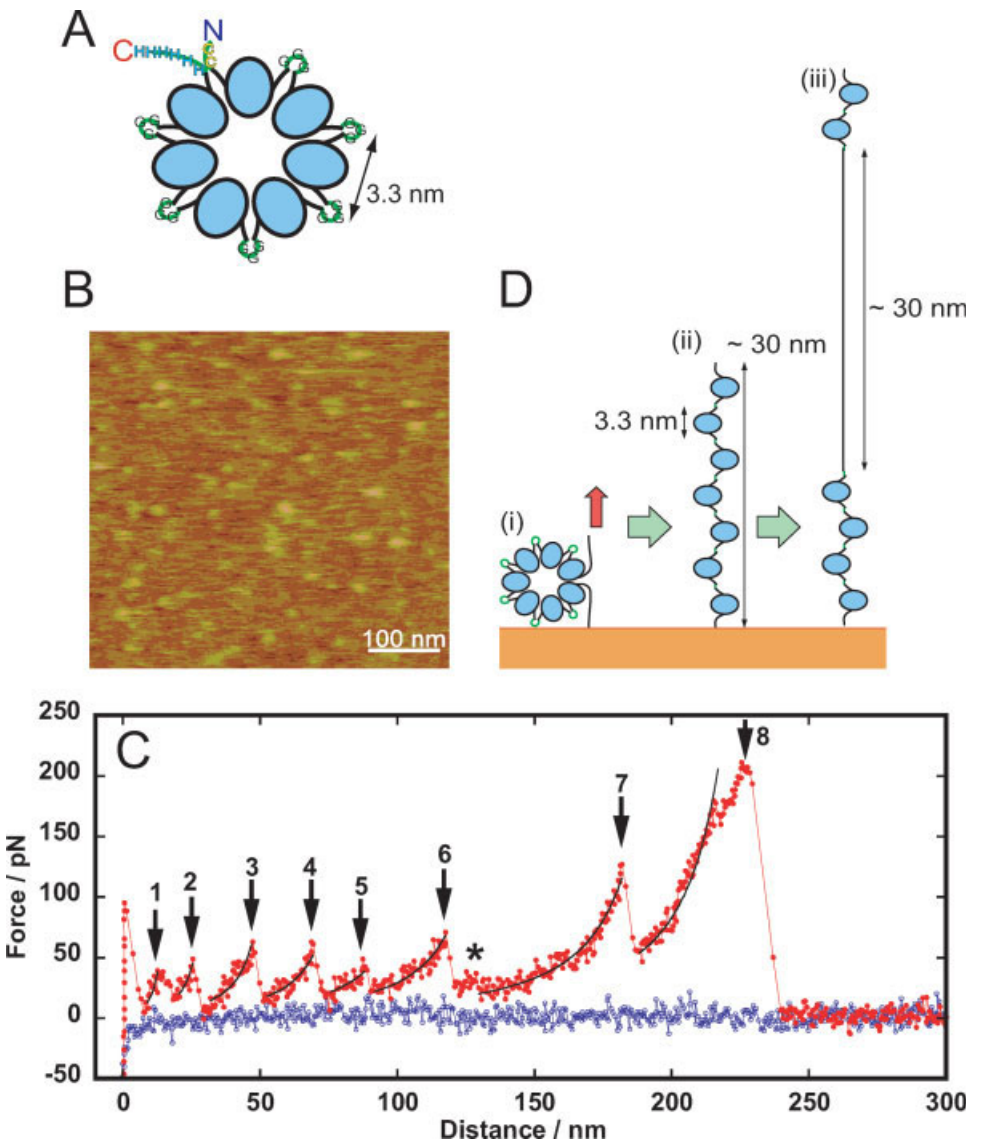

Figure 1. Mechanical unfolding of CH-ESC7. (**A**) Schematic diagram of a CH-ESC7 molecule. A single polypeptide chain variant of GroES was constructed by consecutively linking the C terminus of one subunit to the N terminus of an adjacent subunit with a three-glycine linker. Two cysteine residues and six histidine residues were extended from the N and C termini of this single-chain variant, respectively. (**B**) AFM image of CH-ESC7 immobilized on the mercapto-silanized mica surface. Scale bar, 100 nm. (**C**) Force-extension profile obtained from a mechanical extension experiment with CH-ESC7. The probe approached the mica surface (blue lines with open symbols) and caught a single CH-ESC7 molecule on the surface of its tip. Then the probe withdrew from the surface (red lines with filled symbols). The force exerted on the protein was plotted as a function of the end-to-end distance of the protein. Arrows show major peaks in the profile. Black lines are the best fits of Eq. (1) against corresponding segments of the profile. The asterisk denotes a subtle peak that may be attributed to the sixth unfolding event (see text). (**D**) Model for the mechanical unfolding of CH-ESC7. (i) A CH-ESC7 molecule immobilized on the mica surface was picked up by the AFM probe. (ii) Then the heptamer ring was cleaved by force and resulted in a ~30-nm-long structure. Further mechanical extension resulted in consecutive unfolding of linked-GroES subunits. (iii) Each unfolding event of the subunits contributed to the increase in contour length by ~30 nm.

chain caused by loss of structure.[16] The ascending side of each peak was analyzed according to the worm-like chain model[18] by fitting Eq. (1) to the experimental data. The fitting parameter L_c is the contour length, which represents the length of the unstructured polypeptide chain. The L_c values obtained for the eight significant peaks are listed in Table II. The differences in the L_c values between adjacent peaks (ΔL_c) provide hints to the molecular events that underlie each peak of the force extension profile.

Of a total of seven distinct ΔL_c values, five were clustered at around 30 nm (the average ΔL_c value of the five ΔL_c values was 29 nm, omitting the ΔL_c value of peak **2** to **1** and peak **7** to **6**). The significance of this value may be explained when we calculate the length of a single unfolded GroES monomer. Using a

Table I. *Cochaperonin Activities of GroES and CH-ESC7*

Chaperonin	Relative cochaperonin activities (%)	
	ATPase[a]	Rhodanese refolding[b]
GroEL only	100	8
GroEL and GroES	53	100
GroEL and CH-ESC7	51	96

[a] Relative ATPase activities of GroEL in the presence of GroES or CH-ESC7 are shown as ratios to the ATPase activity of GroEL in the absence of GroES. ATPase activities were measured at 25°C.

[b] Rhodanese was unfolded in 4 *M* Gdn-HCl and refolded in the presence of GroEL and either GroES or CH-ESC7 at 25°C. Rhodanese activities, which were measured after refolding for 60 min, are shown as ratios to the recovered activity of rhodanese refolded in the presence of GroEL and GroES.

value of 0.34 nm as the distance between two adjacent C_α atoms in a polypeptide,[19,20] and considering that GroES is composed of 97 amino acid residues, we arrive at a value of 33 nm (= 97 aa × 0.34 nm) as the theoretical length of a single unfolded GroES monomer. The similarity in the theoretical length and the ΔL_c values suggest that in these five cases (peaks **2**, **3**, **4**, **5**, and **7**) we are observing unfolding of individual GroES subunits.

With regard to the remaining two ΔL_c values that vary significantly from the theoretical length of the GroES subunit, we observed that the ΔL_c value between peaks **7** and **6** (67 nm) was nearly identical to twice the theoretical length of an unfolded GroES subunit. Additionally, we observed a very slight, nearly undetectable peak between peak **7** and peak **6** [asterisk in Fig. 1(C)]. These two observations suggest that in peak **6**, we are observing simultaneous or nearly simultaneous unfolding of two subunits of GroES within a single extension. Although we do not present the data here, in multiple instances of mechanical unfolding of CH-ESC7, we sometimes observed similar extensions in the basal profile that seemed to point toward simultaneous unfolding of GroES subunits. Here, it should be noted that in chemical unfolding experiments, we have determined that the covalent linker between the subunits restricts the mobility of the subunits as a whole but does not change the stability of an individual subunit.[14]

The remaining ΔL_c value, derived from peaks **2** to **1**, was significantly smaller than the theoretical value of unfolded GroES subunit. Although we do not possess additional confirming data, we observed that the net extension of the polymer seen after peak **1**, represented by an L_c value of 34 nm, was reasonably close to the theoretical length of seven intact GroES subunits each linked by a triglycine linker (the distance between the N and C terminus of an intact GroES subunit is estimated at 3.3 nm from X-ray crystallographic measurement,[6] and the length of a single triglycine linker is about 1.0 nm; these two values yield a total of 3.3 nm × 7 + 1.0 nm × 6 = 29.1 nm). From this, we believe that peak **1** represents the disruption of the initial heptameric ring structure of GroES [Fig. 1(D-ii)].

The average unfolding force of a single GroES subunit determined in our experiments was estimated at about 50 pN. This value is small when compared with that of other compact β-barrel proteins, such as the immunoglobulin-like molecules of titin, which unfold at ~200 pN with an extension rate similar to the one in this study.[16,21] In fact, the unfolding force of GroES subunits is rather similar to that of all-α and α + β proteins, such as spectrin (~30 pN at 300 nm s^{-1}),[22] T4 lysozyme (~60 pN at 1000 nm s^{-1}),[19] and barnase (~70 pN at 300 nm s^{-1}).[23] This relatively small unfolding force may be explained by the marginal stability of GroES (2.9 kJ mol^{-1}[24] and 9.6 kJ mol^{-1}[9]) compared with other globular proteins, although thermodynamic stability (i.e., unfolding free energy) of a protein is not directly correlated to its mechanical stability.[25] Moreover, it has been reported that the mechanical stability of proteins is dependent

Table II. *Parameters Obtained from the Force-Extension Profile*

Peak no.[a]	Force (pN)	L_c (nm)[b]	ΔL_c (nm)[c]	N_u[d]	ΔN_u[e]	$\Delta L_c/\Delta N_u$ (nm)
1	34	18				
2	42	34	16	0		
3	56	60	26	1	1	26
4	56	91	31	2	1	31
5	39	122	31	3	1	31
6	66	147	26	4	1	26
7	120	215	67	6	2	34
8	210	245	30	7	1	30
Average	78		32			30

[a] Major peaks in Figure 1(C) are counted.

[b] Calculated for each peak using Eq. (1).

[c] Difference in L_c between two neighboring peaks.

[d] Estimated number of unfolded GroES domains in CH-ESC7. Note that there is no number 5, because the "peak" between peaks **6** and **7** was too small for accurate analysis—its absence would be explained by the nearly simultaneous unfolding of the fifth and sixth subunits.

[e] Difference in N_u between two neighboring peaks.

not only on their structures but also on their stretching configurations.[12,26] In the case of GroES, since the N and C termini of the native subunits are spatially placed in close proximity, an external force is exerted on the subunit in opposite directions from a relatively confined position on the protein surface; the hydrogen bonds that fasten the strands may break easily and peel the β-strands away from the core β-barrel structure upon application of a relatively weak force, resulting in these small unfolding force values.

A noteworthy characteristic of the data that we collected in this study is that we observed the distinctive sawtooth pattern in the extension profile [Fig. 1(C)]. Although we were not able to obtain sufficient traces to allow a more quantitative analysis of the mechanical unfolding of CH-ESC7, we were able to collect multiple extension profiles of the unfolding of this protein and the sawtooth pattern was prevalent in each profile. In the case of CH-ESC7, this characteristic pattern suggests that CH-ESC7 may form an intermediate state where the quaternary structure is disrupted, but individual subunits retain most of their native structure. The existence of folded monomers of GroES and other cpn10 families has been suggested in equilibrium unfolding experiments,[4,9] equilibrium sedimentation experiments,[2] dilution-induced dissociation experiments,[2,8] and kinetic refolding experiments.[24,27] However, this intermediate state was not universally observable.[8,10,24,27] In this study, since we used mechanical denaturation techniques under biologically relevant buffer conditions, where the native structure of cpn10 is favored, the fact that we were able to obtain data that pointed to the existence of stable monomeric forms of GroES during unfolding is of great importance and suggests strongly that the stability of GroES monomers also contributes significantly to the overall stability of the cochaperonin.

In a more general context, mechanical unfolding studies of oligomeric proteins, such as those performed here, offer a viable alternative in the analysis of the folding behavior of complex oligomeric proteins, as it allows us to circumvent some unwanted side reactions that are prone to occur during chemical denaturation experiments (most notably, nonspecific aggregation). It remains to be seen whether similar experiments, applied to other oligomeric proteins, may yield further important insights to oligomeric protein folding and association.

Methods

Proteins

The expression vector of covalently linked GroES (pESC7)[14] was modified as follows to produce a variant of ESC7 suitable for mechanical manipulation. Small DNA fragments were inserted at the *Sma*I and *Nae*I sites of the pESC7 vector, which resulted in the formation of a new vector, pCH-ESC7. Then, the *E. coli* cells were transformed with the pCH-ESC7 vector. The result was that the *E. coli* cells produced CH-ESC7 protein, which has two consecutive cysteine residues and six consecutive histidine residues at its N and C termini, respectively. The protein sequence of this ESC7 variant (CH-ESC7) is MPCCGGG-(*ES1*)-GGG-(*ES2*)-. . .-(*ES7*)-GGGGHHHHHH, where (*ESn*) represents the *n*th native sequence of GroES. The total number of amino acid residues in CH-ESC7 is 714. CH-ESC7 protein was expressed in *E. coli* using the same procedure for ESC7.[14] Judging by SDS-PAGE analysis, some truncated proteins that were possibly caused by protease degradation from the intact, full-length CH-ESC7 were also observed. Although we tried to purify CH-ESC7 using nickel-chelate column chromatography, we could not separate the truncated proteins. Thus, we adopted a purification protocol for ESC7 that includes a heat treatment, in which the truncated proteins are coprecipitated with other thermally unstable proteins.

GroEL and GroES were expressed in *E. coli* and purified according to published protocols.[28]

Assays for cochaperonin activity

Cochaperonin activities of GroES and CH-ESC7 were evaluated by measuring the ATPase activities of GroEL and refolding yields of rhodanese at 25°C in the presence of GroEL and either GroES or CH-ESC7, as described previously.[9]

AFM measurements

Atomic force microscopic measurements were made with a MMAFM-2 scanning microscope driven by a Nanoscope IV controller (Veeco Instruments, Woodbury, NY). AFM micrographs were captured in the AC mode (tapping mode) in PBS [137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 2 m*M* KH_2PO_4 (pH 7.4)]. Silicon-nitride probes with gold coating on both tip and reflex sides (Olympus, Tokyo, Japan) were used to collect measurement data. The stiffness of the cantilevers ranged from 18 to 24 pN nm^{-1}, as estimated from their thermal fluctuations. For analysis of force measurements, 20 pN nm^{-1} was used as the cantilever stiffness. Force-extension profiles were obtained in the force-ramp mode with 500 nm amplitude and 5 s duration (i.e., extension rate was 200 nm s^{-1}) in PBS at 25°C.

Surface modification of mica and protein immobilization

A freshly cleaved mica surface was exposed to the vapor of 0.01% (v/v) (3-mercaptopropyl)triethoxysilane (Avocado Research Chemicals, Lancashire, UK) dissolved in dry toluene for 4 hr. A droplet of 0.1 mg mL^{-1} CH-ESC7 solution in PBS was placed on the mercapto-silanized mica surface for 10 min at room temperature, and the surface was washed several times with PBS.

Data analysis

The length of the protein molecule was calculated from the equation $l = Z - d$, where Z is displacement of the cantilever base against the mica surface and d is deflection of the cantilever. The force applied to the protein molecule was determined from the equation $F = Kd$, where K is stiffness of the cantilever. Then, the relations between l and F were plotted as force-extension profiles.

The force-extension profiles were analyzed by fitting the worm-like-chain model to the experimental data[18]:

$$F = \frac{k_B T}{p}\left(\frac{1}{4(1 - l/L_c)^2} + \frac{l}{L_c} - \frac{1}{4}\right) \quad (1)$$

where k_B is Boltzmann's constant, T is temperature, p is persistence length, and L_c is contour length. We used $T = 298$ K (25°C) to represent the temperature during our experiment. Equation 1 was fitted to the ascending sides of the force-extension profiles by using the nonlinear least-squares method.

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