doi:10.1016/j.jmb.2012.05.026 J. Mol. Biol. (2012) 422, 291–299

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Flexibility of GroES Mobile Loop Is Required for Efficient Chaperonin Function

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Received 20 April 2012; accepted 15 May 2012
Available online
25 May 2012

Edited by J. Buchner

Keywords:
GroEL;
GroES;
chaperonin;
disulfide bond;
mobile loop

Chaperonin GroEL and its partner GroES assist the folding of nascent and stress-damaged proteins in an ATP-dependent manner. Free GroES has a flexible "mobile loop" and binds to GroEL through the residues at the tip of the loop, capping the central cavity of GroEL to provide the substrate polypeptide a cage for secure in-cage folding. Here, we show that restriction of the flexibility of the loop by a disulfide cross-linking between cysteines within the loop results in the inefficient formation of a stable GroEL–polypeptide–GroES ternary complex and inefficient folding. Then, we generated substrate proteins with enhanced binding affinity to GroEL by fusion of one or two SBP (strongly binding peptide for GroEL) sequences and examined the effect of disulfide cross-linking on the assisted folding. The results indicate that the higher the binding affinity of the substrate polypeptide to GroEL, the greater the contribution of the mobile loop flexibility to efficient in-cage folding. It is likely that the flexibility helps GroES capture GroEL's binding sites that are already occupied by the substrate polypeptide with various binding modes.

Introduction

Escherichia coli chaperonin GroEL facilitates the folding of nascent and stress-damaged proteins in an ATP-dependent manner[1–4] and is essential for cell viability.[5] It is a large cylindrical protein complex comprising two heptameric rings of identical 57-kDa subunits stacked back to back.[6] GroEL binds an unfolded polypeptide to its apical domains surrounding the opening of the central cavities and forms a stable GroEL–polypeptide binary complex.[7–11] Upon ATP binding, GroEL undergoes conformational transition[12] that enables binding of co-chaperone GroES, a disk-shaped homo-heptamer of 10-kDa subunits,[13] and the central cavity of GroEL is capped by GroES to make an enclosed cage. Substrate polypeptide is entrapped into the cage[14,15] with a part of the polypeptide still being tethered to the GroEL apical domain.[16] Then, the entire polypeptide is completely released into the cage, where it gains native conformation rapidly (in-cage folding). In some cases, dependent on substrate polypeptide, a fraction of the polypeptide escapes to the environment and undergoes free folding (or is captured again by GroEL).

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Abbreviations used: 2D, two-dimensional; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; GFP, green fluorescent protein; IPMDH, 3-isopropylmalate dehydrogenase; NOESY, nuclear Overhauser effect spectroscopy; SBP, strongly binding peptide (SWMTTPWGFLHP); TOCSY, total correlation spectroscopy.

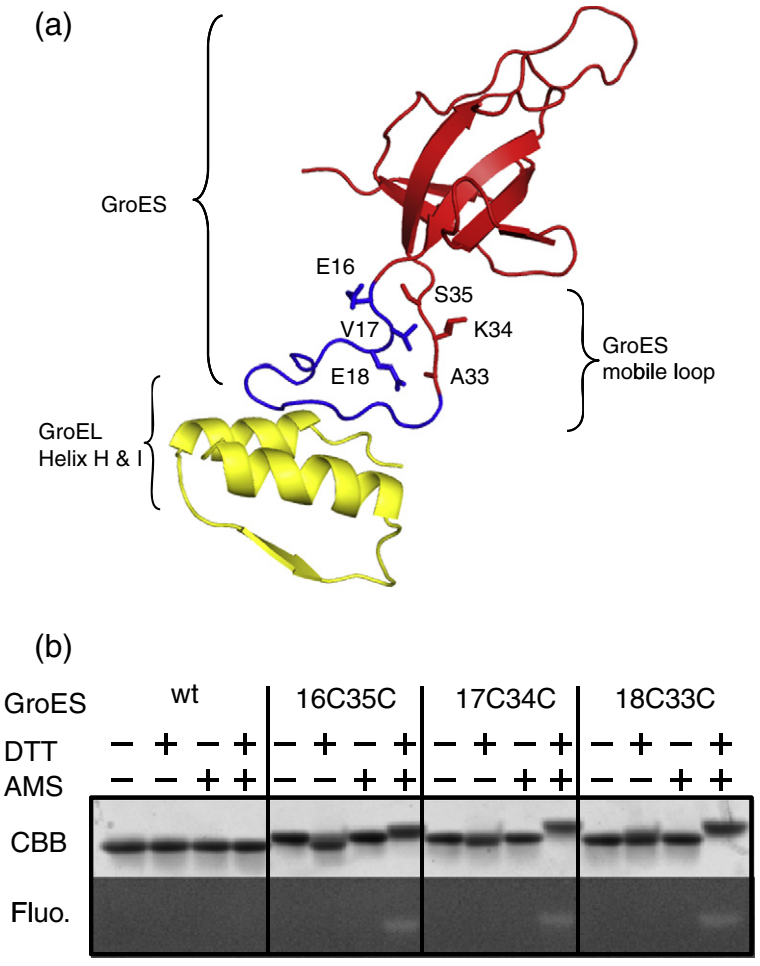

Fig. 1. GroES mobile loop in the GroEL–GroES crystal structure. (a) Structure of GroES subunit associated with helices H and I of GroEL (yellow) in the crystal structure of the GroEL–GroES complex (PDB: 1AON[19]). In the GroES heptamer crystal structure, the red region is visible in all seven GroES subunits, but the blue region is visible only in one GroES subunit. A pair of residues shown in stick model (E16/S35, V17/K34, E18/A33) were replaced with cysteines and three GroES$_{(2C)}$s [GroES$_{(16C/35C)}$, GroES$_{(17C/34C)}$, and GroES$_{(18C/33C)}$] were generated. (b) Disulfide bond formation in GroES$_{(2C)}$s and its reduction by DTT. Remaining sulfhydryl groups were labeled by AMS and analyzed with SDS-PAGE. Protein was detected by Coomassie Brilliant Blue staining (upper panel) and AMS-bound protein was detected by fluorescence (lower panel). Band shift and AMS fluorescence were observed only in DTT-reduced and AMS-treated samples.

Free GroES subunit contains a highly flexible region named "mobile loop".[17] In the crystal of the GroES heptamer, this region (amino acid residues 16–32) is disordered in six subunits.[13] Upon binding to GroEL, the mobile loops of all seven GroES subunits are fixed to the β-hairpin structure.[18,19] Despite these early studies, the functional contribution of the flexibility of the mobile loop to the assisted folding remains unclear. Here, we have made GroES mutants whose flexibility of the mobile loop can be restricted by the formation of a disulfide bond between two cysteine residues introduced into the opposite side of the mobile loop. Tests on co-chaperone activity of GroES having a cross-link in the mobile loop show that the flexibility of the mobile loop contributes to the formation of a stable GroEL–polypeptide–GroES complex and for efficient in-cage folding. Using substrate polypeptides fused with the "strongly binding peptide" (SBP) that has high affinity to the apical domain of GroEL,[20] we show that contribution of the mobile loop flexibility of GroES is more pronounced as the substrate polypeptide has higher affinity to GroEL.

Results

Specific cross-linking in GroES mobile loop

GroES binds to GroEL via its mobile loop.[17] Three pairs of amino acid residues positioning in the opposite side in the mobile loop, E16/S35, V17/K34, and E18/A33, were individually replaced with cysteine residues. In the heptameric GroES crystal,[13] A33, K34, and S35 are structurally ordered in all seven subunits while E16, V17, and E18 are disordered in six subunits (Fig. 1a).[13] Since wt-GroES contains no cysteine, pairs of the cysteines introduced into these mutants [collectively termed GroES$_{(2C)}$s] are expected to make a disulfide bond by oxidation that restricts the flexibility of the disordered residue. Note that these six residues do not have direct interaction with GroEL in the GroEL–GroES crystal.[19] Indeed, a mutant GroES replacing all of these six residues by alanines is indicated to be functional because it can complement wt-GroES in *E. coli* cells (not shown). Oxidation of cysteines was assessed by the amount of remaining non-

oxidized cysteines. Chemical labeling of cysteine with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) resulted in the production of a fluorescent protein band with a decreased electrophoretic mobility in SDS-PAGE.[21] The introduced cysteines in the purified $GroES_{(16C/35C)}$ and $GroES_{(18C/33C)}$ were fully oxidized. Cysteines in the purified $GroES_{(17C/34C)}$ were partly oxidized, which we treated with an oxidizing reagent, diamide, to obtain fully oxidized $GroES_{(17C/34C)}$. These fully oxidized $GroES_{(2C)}$s were used as oxidized samples hereafter. The oxidized $GroES_{(2C)}$s were completely reduced by DTT (Fig. 1b). DTT-treated $GroES_{(2C)}$s were used as reduced samples and their assays were all carried out in the presence of DTT. There was no dimer in the oxidized $GroES_{(2C)}$s (not shown), ensuring that disulfide cross-linking occurred in the same GroES subunit molecule. All $GroES_{(2C)}$s can complement wt-GroES in *E. coli* cells (not shown). Since *E. coli* cytosol has a reductive environment and $GroES_{(2C)}$s should exist as a reduced form, this result indicates that reduced $GroES_{(2C)}$s are functional *in vivo*.

Binding to GroEL

Binding of oxidized and reduced $GroES_{(2C)}$s to GroEL was examined (Fig. 2a). Cy3-labeled GroES was mixed with GroEL in the presence of ATP and analyzed by gel filtration monitored with Cy3 fluorescence. All $GroES_{(2C)}$s were co-eluted with GroEL irrespective of their redox state, suggesting that flexibility of GroES mobile loop contributes little to GroES binding to GroEL. As expected, the ATPase activity of GroEL decreased to ~25% by the presence of wt-GroES and reduced and oxidized $GroES_{(2C)}$s (not shown). Binding to the GroEL–rhodanese binary complex was also tested (Fig. 2b). $GroES_{(16C/35C)}$ and $GroES_{(17C/34C)}$ were co-eluted with the binary complex regardless of their redox state. For $GroES_{(18C/33C)}$, however, the oxidized form showed poorer binding to the GroEL–rhodanese binary complex than the reduced form. Consistently, rhodanese in the complex was susceptive to protease digestion when oxidized $GroES_{(18C/33C)}$ was added while it was protected in the case of reduced $GroES_{(18C/33C)}$ (not shown). It appears that oxidized $GroES_{(18C/33C)}$ loses flexibility of its mobile loop most completely among $GroES_{(2C)}$s and tends to fail binding to the GroEL–rhodanese binary complex.

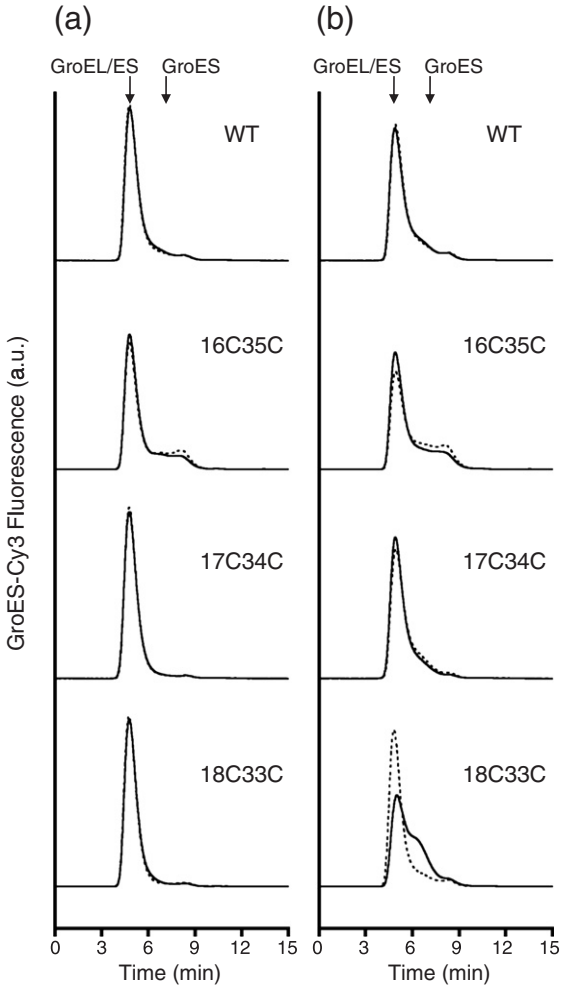

Fig. 2. Binding of reduced and oxidized $GroES_{(2C)}$s to GroEL and the GroEL–rhodanese complex. Binding of Cy3-labeled wt-GroES and $GroES_{(2C)}$s to (a) GroEL or (b) the GroEL–rhodanese binary complex was assessed by gel-filtration HPLC. The GroEL–rhodanese binary complex was generated by heat treatment. Reduced GroES-Cy3 (broken line) and oxidized GroES-Cy3 (continuous line) were mixed with GroEL at a 1:1 ratio in the presence of 0.5 mM ATP and were applied to gel-filtration HPLC equilibrated with 0.2 mM ATP. Elution was monitored with fluorescence of GroES-Cy3. The GroEL–GroES complex and free GroES were eluted at 5 min and 7 min (shown by arrows), respectively. Each elution pattern was normalized so that the total peak area became equal among elution. Detailed experimental conditions are described under Materials and Methods.

Co-chaperone activity of reduced and oxidized $GroES_{(2C)}$s in assisted folding of rhodanese

GroEL/GroES-assisted folding of rhodanese, assessed by recovery of rhodanese activity, was compared between wild-type and $GroES_{(2C)}$s under reduced and oxidized conditions. Bovine rhodanese contains four cysteine residues, and its folding assay requires DTT to prevent nonproductive disulfide bond formation. Then, we used $rhodanese_{(1C)}$, in which all cysteines, except for an essential Cys242 in the active site,[22,23] were replaced with serine. This $rhodanese_{(1C)}$ recovered activity in the absence of DTT, though the yield was slightly smaller than in the presence of DTT. Folding of $rhodanese_{(1C)}$ did

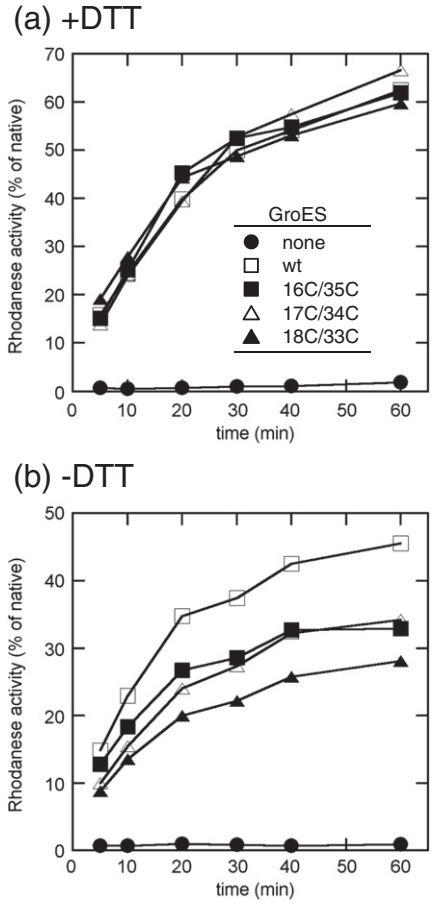

Fig. 3. Co-chaperone activity of reduced and oxidized $GroES_{(2C)}$s in GroEL/GroES-assisted folding of $rhodanese_{(1C)}$. The GroEL–$rhodanese_{(1C)}$ binary complex was generated by heat treatment in the presence (a) or absence (b) of DTT. Recovery of rhodanese activity was measured at indicated times after the addition of ATP. Rhodanese activity was expressed as the percentage of that of native $rhodanese_{(1C)}$. The same experiments were repeated four times, and the data were averaged. Detailed experimental conditions are described under Materials and Methods.

not occur when GroES was omitted from the solution (Fig. 3), confirming the indispensable role of GroES as a co-chaperone in rhodanese folding. Recovery time courses of rhodanese activity in the presence of DTT were almost the same between $GroES_{(2C)}$s and wt-GroES (Fig. 3a). On the contrary, in the absence of DTT, they were significantly different between $GroES_{(2C)}$s and wt-GroES; recovery yield was 70% [$GroES_{(16C/35C)}$ and $GroES_{(17C/34C)}$] and 60% [$GroES_{(18C/33C)}$] of wt-GroES. These results indicate that the yield of the in-cage folding decreases when the flexibility of the mobile loop is restricted by cross-linking. For $GroES_{(16C/35C)}$ and $GroES_{(17C/34C)}$, significant change in binding to the binary complex is not detected between reduced and oxidized forms (Fig. 2b), but folding becomes less efficient by oxidation.

Assisted folding of IPMDHs with increased binding affinity to GroEL

We generated 3-isopropylmalate dehydrogenases (IPMDHs) that have different binding affinity to GroEL and compared the co-chaperone activity of reduced and oxidized $GroES_{(2C)}$s in the folding of these IPMDHs. IPMDH from *Thermus thermophilus* was used since it contains no cysteine residue and folding is not affected by redox condition. IPMDH was fused with the SBP sequence to the C-terminus (IPMDH-SBP) or to both N- and C-termini (SBP-IPMDH-SBP). SBP is a peptide of 12 residues, which was discovered as a strongly binding peptide for GroEL,[20] and it is expected that binding affinity to GroEL increases in the order IPMDH<IPMDH-SBP<SBP-IPMDH-SBP.[24] IPMDH can fold spontaneously,[25] while folding of IPMDH-SBP and SBP-IPMDH-SBP is strictly dependent on GroEL and GroES. As a co-chaperone, $GroES_{(2C)}$s, irrespective of redox state, were as effective as wt-GroES in the folding of IPMDH and IPMDH-SBP (Fig. 4a and b). When SBP-IPMDH-SBP was used as a substrate protein, all $GroES_{(2C)}$s and even wt-GroES showed a decreased level of folding yield (Fig. 4c). Among them, the effect of redox condition was clearly seen only in the assisted folding by GroEL and $GroES_{(18C/33C)}$; folding yield by the oxidized $GroES_{(18C/33C)}$ was only ~20% of that by the reduced $GroES_{(18C/33C)}$. Thus, the defect of oxidized $GroES_{(18C/33C)}$ in assisted folding becomes evident when a substrate polypeptide has stronger binding affinity to GroEL.

Co-chaperone activity of reduced and oxidized $GroES_{(18C/33C)}$ in SBP-fused GFP folding

Among the three $GroES_{(2C)}$s, $GroES_{(18C/33C)}$ exhibited the most significant redox dependency in binding to the GroEL–rhodanese binary complex and in the assisted folding of rhodanese and SBP-IPMDH-SBP. Then, the co-chaperone activity of $GroES_{(18C/33C)}$ was further examined in the folding of SBP-fused green fluorescent protein (GFP) (Fig. 5). Folding of GFP can be monitored by its fluorescence recovery in real time.[26] In this series of experiments, a single-ring mutant of GroEL containing slow ATP-hydrolyzing mutation D398A, $SR1_{D398A}$, was used[14,27] to see a single turnover of the in-cage folding by avoiding complication caused by the interaction between two GroEL heptamer rings. The $SR1_{D398A}$–GFP binary complex was prepared by the dilution of acid-denatured GFP into the $SR1_{D398A}$ solution, and the reaction was initiated by the addition of ATP. The observed

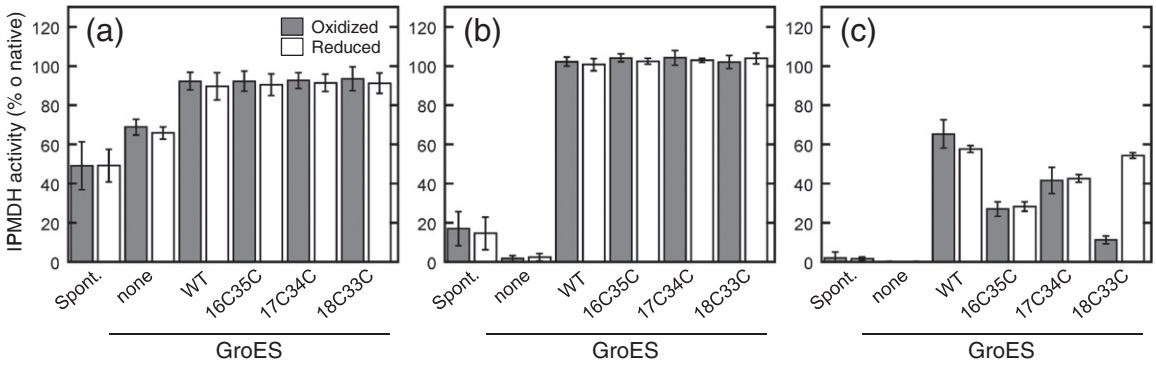

Fig. 4. Co-chaperone activity of reduced and oxidized $GroES_{(2C)}$s in GroEL/GroES-assisted folding of (a) IPMDH, (b) IPMDH-SBP, and (c) SBP-IPMDH-SBP. The binary complex of GroEL with IPMDHs was generated by dilution of denatured IPMDH in 4 M guanidine–HCl into the buffer containing GroEL. Recovery of IPMDH activity was measured at 20 min after the addition of ATP. Spontaneous folding (Spont.) was measured by dilution of denatured IPMDH into the buffer containing 0.4 mg/ml bovine serum albumin. The same experiments were repeated three times, and the data were averaged. IPMDH activity was expressed as the percentage of the native IPMDH. Detailed experimental conditions are described under Materials and Methods.

folding represented the in-cage folding since trap-GroEL[28] was added prior to ATP addition to capture any unfolded GFP that has escaped from the cage during the folding reaction. Spontaneous folding of GFP, GFP-SBP, and SBP-GFP-SBP became slower in this order (not shown), and the folding assisted by $SR1_{D398A}$ and wt-GroES (Fig. 5, continuous lines) also proceeded at rates similar to the corresponding spontaneous folding. For all GFPs, folding assisted by $SR1_{D398A}$ and reduced $GroES_{(18C/33C)}$ proceeded in a time course similar to that of folding assisted by $SR1_{D398A}$ and wt-GroES (Fig. 5, dotted lines). On the contrary, folding assisted by $SR1_{D398A}$ and oxidized $GroES_{(18C/33C)}$ occurred only slowly with GFP-SBP and extremely slowly with SBP-GFP-SBP (Fig. 5b and c, broken line). Even though the rates are different between oxidized and reduced states of $GroES_{(18C/33C)}$, final yield of folding reached nearly the same level. These results again indicate that the effect of cross-linking becomes more evident as the affinity of substrate polypeptide to GroEL increases.

NMR analysis of flexibility of the mobile loop

In the above experiments, we assumed that the disulfide bond between residues 18 and 33 in the oxidized $GroES_{(18C/33C)}$ restricts the movement of the mobile loop. To confirm this, we utilized nuclear magnetic resonance (NMR) spectroscopy and

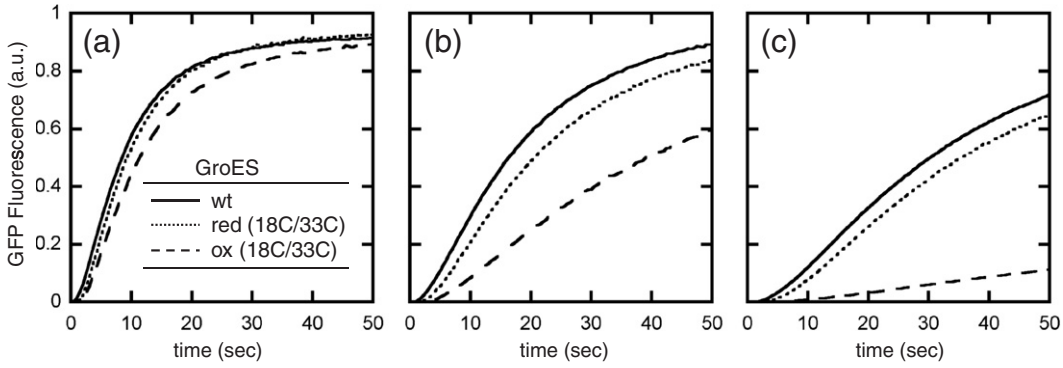

Fig. 5. Time courses of in-cage folding of (a) GFP, (b) GFP-SBP, and (c) SBP-GFP-SBP by $SR1_{D398A}$ and reduced or oxidized $GroES_{(18C/33C)}$. Denatured GFPs were diluted into the dilution buffer containing a single-ring GroEL ($SR1_{D398A}$) that allowed single turnover of folding. The dilution buffer also contained wt-GroES (continuous line), reduced $GroES_{(18C/33C)}$ (dotted line), or oxidized $GroES_{(18C/33C)}$ (broken line). To observe only the in-cage folding, trap-GroEL was added prior to ATP addition to prevent spontaneous folding in the medium. Folding was initiated by adding ATP at 0 s. The fluorescence intensity of each curve was normalized as a fraction of the recovered fluorescence intensity at the saturated point. Detailed experimental conditions are described under Materials and Methods.

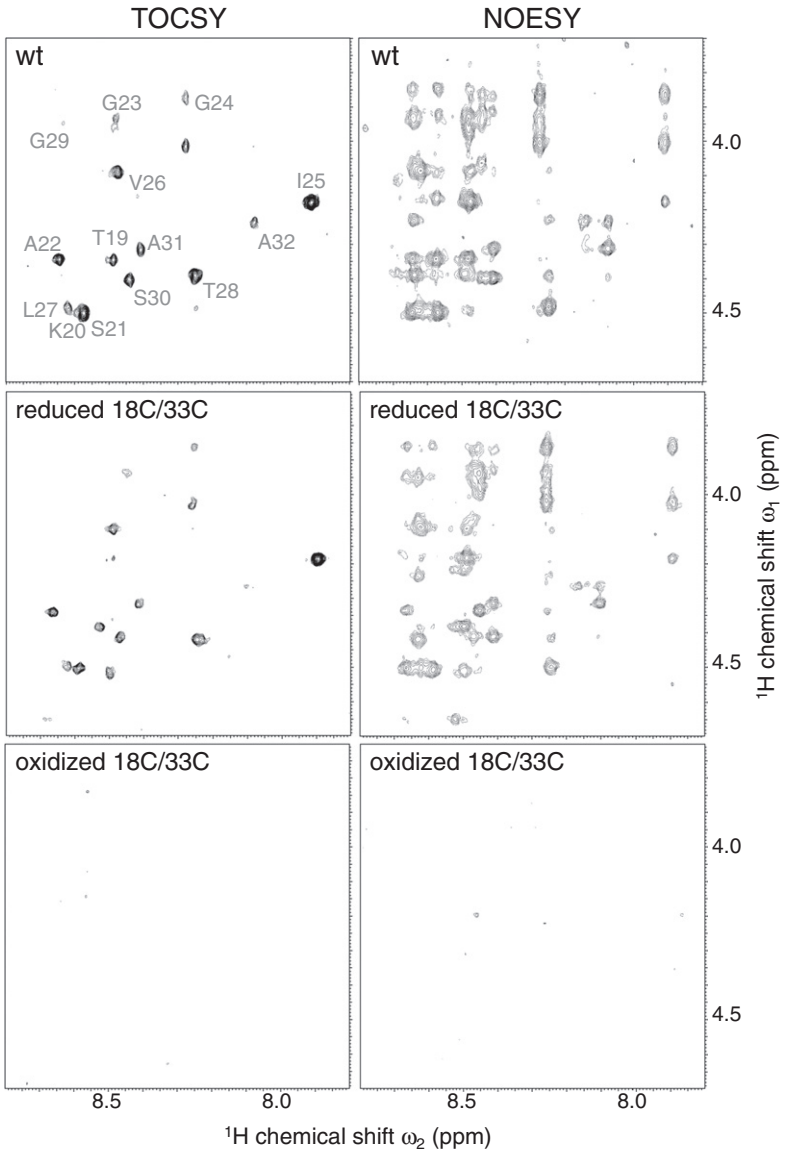

Fig. 6. NMR spectra of GroES. 2D TOCSY (left) and NOESY (right) spectra of wt-GroES (top), reduced $GroES_{(18C/33C)}$ (middle), and oxidized $GroES_{(18C/33C)}$ (bottom) are presented. For each spectrum, a fingerprint region (amide protons in the direct dimension and α-protons in the indirect dimension) is displayed as in Ref. 17. The peaks that were assigned by referencing the result of Ref. 17 are labeled with their amino acid names and sequence numbers.

measured the two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) spectra of wt-GroES, reduced $GroES_{(18C/33C)}$, and oxidized $GroES_{(18C/33C)}$. As reported for wt-GroES,[17] reduced $GroES_{(18C/33C)}$ exhibited well-resolved cross peaks in both spectra corresponding to the mobile region that spans amino acids 18–32 in the GroES sequence (Fig. 6). Most of the cross peaks of reduced $GroES_{(18C/33C)}$ were observed at resonance positions similar to those of wt-GroES. As expected, the spectra of wt-GroES were almost the same as that reported in Ref. 17, based on which most cross peaks were assigned. In contrast, oxidized $GroES_{(18C/33C)}$ showed only broadened signals almost beyond detection in the measurement condition that was, except for the addition of deuterated DTT, the same as that adopted for the reduced $GroES_{(18C/33C)}$. In liquid-state NMR, peaks derived from small molecules or flexible parts are generally observable, as demonstrated in the mobile loop region of wt-GroES and reduced $GroES_{(18C/33C)}$, but those derived from large molecules, such as the remaining dominant part of GroES, are too broadened to be detected. This result justified the restricted mobility of the mobile loop in oxidized $GroES_{(18C/33C)}$.

Discussion

In this article, we made three GroES mutants in which flexibility of the mobile loop would be restricted by a disulfide bond between a disordered residue and an ordered residue in the mobile loop. The most effective is a disulfide bond between introduced cysteine residues at 18 C and 33 C,

which are located most nearly to the tip of the mobile loop (Fig. 1a). Oxidized $GroES_{(18C/33C)}$ can bind GroEL normally but tends to fail to bind to the GroEL–polypeptide binary complex. It has been known that residues of GroEL involved in GroES binding largely overlapped with those involved in polypeptide binding. Therefore, for GroES to bind to the binary complex, GroES should deprive the substrate polypeptide of the binding sites on GroEL. Flexibility of the mobile loop can give GroES an advantage over the polypeptide in competing for the common binding sites. It was reported that even the same substrate polypeptide can bind to GroEL with various binding manners and generates micro-heterogeneity among the binding sites.[29] Flexibility of the mobile loop possibly contributes to a fine-tuning of binding mode to fit the heterogeneous binding sites.

Yield and time course of the assisted folding are also affected by cross-linking. In the case of rhodanese, not only the oxidized form of $GroES_{(18C/33C)}$ but also $GroES_{(17C/32C)}$ and $GroES_{(16C/31C)}$ showed decreased folding yields, reflecting an inefficient step after the ternary complex formation, which is probably the step of encapsulation of the substrate polypeptide into the cage. In the case of IPMDH and GFP, the effect of cross-linking was more pronounced as the binding affinity of the substrate polypeptide to GroEL increased by SBP fusion. It is tempting to assume that the flexibility of the mobile loop contributes when GroES forcibly occupies the binding sites of GroEL that are preoccupied by a strongly bound substrate polypeptide. It is also plausible that the SBP fusion substrates simply do not release with sufficient frequency from the apical domains to allow access for the flexibility-restricted loop of $GroES_{(18C/33C)}$. We suggest, altogether, that the flexibility of the mobile loop helps the residues at the tip of the loop to adjust their conformations to fit the various surfaces of the GroEL–substrate polypeptide complex, inducing the release of the substrate polypeptide into the cage.

Materials and Methods

Reagents and proteins

ATP and NAD^+ were from Sigma. DL-threo-3-Isopropylmalic acid was from Wako. Mutant proteins were generated by site-directed mutagenesis using the PrimeSTAR mutagenesis basal kit from Takara. Wt- and mutant GroEL and bovine rhodanese were expressed and purified as previously described.[30,31] Wt-GroES and mutants were purified as previously described[32] except that DTT was removed throughout the isolation procedures. The redox state of $GroES_{(2C)}$s was assessed as follows. Purified GroES (5 μM) was incubated in HKM buffer (25 mM Hepes–KOH, pH 7.4, 5 mM $MgCl_2$, and 100 mM KCl) with or without 10 mM DTT and incubated for 1 h at 28 °C. After incubation, GroES was precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone, and finally dissolved in 50 mM Tris–HCl (pH 7.4), 1% SDS, and 10 mM AMS.[21] The protein samples were then analyzed with 14% non-reducing SDS-PAGE. AMS-labeled GroES was detected by AMS fluorescence and then the proteins were stained by Coomassie Brilliant Blue. $GroES_{(16C/35C)}$ and $GroES_{(18C/33C)}$ were purified as a 100% oxidized form but about half of purified $GroES_{(17C/34C)}$ exist as a reduced form. Then, purified $GroES_{(17C/34C)}$ (200 μM) was incubated with an oxidizing reagent, 1 mM diamide, in HKM buffer for 1.5 h and fully oxidized $GroES_{(17C/34C)}$ was obtained. In this article, oxidized $GroES_{(2C)}$s mean the naturally oxidized $GroES_{(16C/35C)}$ and $GroES_{(18C/33C)}$ and the diamide-oxidized $GroES_{(17C/34C)}$. Upon exposure to 10 mM DTT, $GroES_{(2C)}$s were reduced completely and we considered them as reduced $GroES_{(2C)}$s. The reaction mixtures for the assay of reduced $GroES_{(2C)}$s always included 5 mM DTT to prevent reoxidation. IPMDH from *T. thermophilus* was expressed in *E. coli* and purified as previously described.[33] GFP (S65T), simply referred to as GFP in this report, was expressed in *E. coli* strain BL21(DE3). Expression of GFP was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside at $A_{600}=0.5$, and the culture was continued at 15 °C for 18 h. Cells were harvested, sonicated in buffer [25 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM DTT, 30% saturated ammonium sulfate, and a Complete protease inhibitor mixture tablet (Roche Applied Science)], and centrifuged. The supernatant was applied to a hydrophobic column (Butyl-650M TOYOPEARL) and eluted with a 30–0% saturated ammonium sulfate linear gradient. The fractions containing GFP was combined and dialyzed to 100-fold volume of 25 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, and 1 mM DTT. The dialyzed solution was applied to an anion-exchange column (DEAE-650M TOYOPEARL) and eluted with a 0–0.3 M NaCl linear gradient. Purified GFP was dialyzed to 50 mM Tris–HCl, pH 7.5, and 50 mM KCl and concentrated by Amicon Ultra (Millipore). The 12-amino-acid sequence (SWMTTPWGFLHP) of SBP was fused to the C-terminus or to both termini of IPMDH and GFP by PCR method. One glycine residue was inserted between SBP and each protein. SBP-fused GFP and IPMDH were similarly expressed and purified to wild type except that heat treatment was skipped in the case of SBP-IPMDH-SBP because of its heat lability. Cy3-labeled GroES (GroES-Cy3) was prepared as previously described.[34,35]

GroEL–GroES binding assays with gel filtration

An aliquot (20 μl) of the solution containing GroES-Cy3 (1.0 μM), GroEL (1.0 μM), and ATP (0.5 mM) in HKM buffer was applied to a column (Superdex200 5/150 GL, GE Healthcare) equilibrated with HKM buffer containing 50 mM Na_2SO_4 and 0.2 mM ATP at a flow rate of 0.3 ml/min. Binding of GroES-Cy3 to the GroEL–rhodanese binary complex was examined as follows. A fivefold molar excess of rhodanese was mixed with GroEL (final concentration, 2.0 μM) in HKM buffer. The mixture was incubated at 60 °C for 15 min to saturate GroEL's binding sites by denatured rhodanese, and

aggregates were removed by centrifugation (15,000*g*, 5 min). The solution containing the GroEL–rhodanese binary complex was mixed with GroES-Cy3 and ATP (final concentrations, 1.0 μM, 1.0 μM, and 0.5 mM, respectively) and an aliquot (20 μl) was applied to the column. The elution was monitored with an in-line fluorometer (excitation at 550 nm, emission at 570 nm).

Folding assay

A rhodanese$_{(1C)}$ mutant (C63S/C254S/C263S) (1.0 μM) and GroEL (1.0 μM) in HKM buffer were incubated at 65 °C for 15 min to form the GroEL–rhodanese$_{(1C)}$ binary complex. Folding reaction was initiated by mixing the GroEL–rhodanese solution with an equal volume of HKM buffer containing 4 μM GroES, 40 mM $Na_2S_2O_3$, 8 mM ATP, and 5 mM DTT when indicated. The mixture was incubated at 25 °C and the folding was quenched at the times indicated. Recovered rhodanese activity was measured as previously described.[36] For the IPMDH folding assay, 50 μM IPMDH from *T. thermophilus* was denatured in 4 M guanidine–HCl at 25 °C for over 5 h. Denatured IPMDH was diluted 100-fold into HKM buffer containing 1 μM GroEL and, in the case of reduced samples, 5 mM DTT. After incubation for 20 min at 37 °C, 4 μM GroES was added. The folding reaction was initiated by the addition of 2 mM ATP, and the solution was incubated at 37 °C. Recovery of IPMDH activity after a 20-min incubation was measured as follows. An aliquot (20 μl) of the solution was added to 1 ml of the IPMDH assay mixture (100 mM potassium phosphate, pH 7.8, 1 M KCl, 1 mM $MgCl_2$, 0.8 mM NAD^+, and 0.4 mM DL-threo-3-isopropylmalic acid) and the increase in the absorbance at 340 nm was monitored at 60 °C.[37] For the GFP folding assay, an acid-denatured GFP solution (5 μM) was diluted 100-fold into HKM buffer containing 200 nM $SR1_{D398A}$ and 1 μM GroES. After 10 min, trap-GroEL ($GroEL_{N265A/D398A}$) was added to a final concentration of 0.5 μM. ATP (1 mM) was added and GFP fluorescence intensity was monitored.

NMR spectroscopy

NMR spectra were recorded at 283 K with a Bruker Avance-III 950-MHz spectrometer equipped with a z-gradient $^1H/^{13}C/^{15}N$ cryogenic probe (TCI). The samples were concentrated to about 0.2–0.3 mM (subunits) in a 20-mM sodium phosphate buffer (pH 6.0) containing 10% ^2H_2O for the NMR lock. Reduced GroES$_{(18C/33C)}$ was prepared by adding 10 mM deuterated DTT to oxidized GroES$_{(18C/33C)}$. In 2D NOESY and TOCSY experiments, the mixing times were set at 200 and 41.4 ms, respectively. The direct ^1H dimension was acquired with a spectral width of 18 ppm with 2048 complex points centered at 4.95 ppm, and the indirect dimension was acquired using the TPPI-States manner with a spectral width of 11 ppm with 600 (wt-GroES) or 800 [reduced and oxidized GroES$_{(18C/33C)}$] complex points. The interscan delay was set at 2.15 s, and 8 (wt-GroES) or 16 [reduced and oxidized GroES$_{(18C/33C)}$] scans were accumulated for each free induction decay. The large water signal was suppressed using the WATERGATE method in the pulse sequences. The NMR data were processed using NMRPipe software package.[38]

Acknowledgement

We thank Dr. T. Hisabori for instruments and valuable discussion. An expression plasmid pET23c-*T*IPMDH for IPMDH was a kind gift from Dr. Y.H. Watanabe. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (No. 19058004 to M.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

1. Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A. & Horwich, A. L. (1998). Structure and function in GroEL-mediated protein folding. *Annu. Rev. Biochem.* **67**, 581–608.
2. Thirumalai, D. & Lorimer, G. H. (2001). Chaperonin-mediated protein folding. *Annu. Rev. Biophys. Biomol. Struct.* **30**, 245–269.
3. Bukau, B. & Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, **92**, 351–366.
4. Jewett, A. I. & Shea, J. E. (2010). Reconciling theories of chaperonin accelerated folding with experimental evidence. *Cell. Mol. Life Sci.* **67**, 255–276.
5. Fayet, O., Ziegelhoffer, T. & Georgopoulos, C. (1989). The groES and groEL heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**, 1379–1385.
6. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, **371**, 578–586.
7. Viitanen, P. V., Gatenby, A. A. & Lorimer, G. H. (1992). Purified chaperonin 60 (groEL) interacts with the nonnative states of a multitude of *Escherichia coli* proteins. *Protein Sci.* **1**, 363–369.
8. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N. & Furtak, K. (1993). Folding in vivo of bacterial cytoplasmic proteins: role of GroEL. *Cell*, **74**, 909–917.
9. Fenton, W. A., Kashi, Y., Furtak, K. & Horwich, A. L. (1994). Residues in chaperonin GroEL required for polypeptide binding and release. *Nature*, **371**, 614–619.
10. Ewalt, K. L., Hendrick, J. P., Houry, W. A. & Hartl, F. U. (1997). In vivo observation of polypeptide flux through the bacterial chaperonin system. *Cell*, **90**, 491–500.
11. Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F. & Hartl, F. U. (1999). Identification of in vivo substrates of the chaperonin GroEL. *Nature*, **402**, 147–154.
12. Nojima, T. & Yoshida, M. (2009). Probing open conformation of GroEL rings by cross-linking reveals single and double open ring structures of GroEL in ADP and ATP. *J. Biol. Chem.* **284**, 22834–22839.
13. Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. & Deisenhofer, J. (1996). The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature*, **379**, 37–45.
14. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K. *et al.* (1995). Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell*, **83**, 577–587.

15. Mayhew, M., da Silva, A. C., Martin, J., Erdjument-Bromage, H., Tempst, P. & Hartl, F. U. (1996). Protein folding in the central cavity of the GroEL–GroES chaperonin complex. *Nature*, **379**, 420–426.
16. Motojima, F. & Yoshida, M. (2010). Polypeptide in the chaperonin cage partly protrudes out and then folds inside or escapes outside. *EMBO J.* **29**, 4008–4019.
17. Landry, S. J., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C. & Gierasch, L. M. (1993). Characterization of a functionally important mobile domain of GroES. *Nature*, **364**, 255–258.
18. Shewmaker, F., Maskos, K., Simmerling, C. & Landry, S. J. (2001). The disordered mobile loop of GroES folds into a defined beta-hairpin upon binding GroEL. *J. Biol. Chem.* **276**, 31257–31264.
19. Xu, Z., Horwich, A. L. & Sigler, P. B. (1997). The crystal structure of the asymmetric GroEL–GroES–(ADP)7 chaperonin complex. *Nature*, **388**, 741–750.
20. Chen, L. & Sigler, P. B. (1999). The crystal structure of a GroEL/peptide complex: plasticity as a basis for substrate diversity. *Cell*, **99**, 757–768.
21. Motohashi, K. & Hisabori, T. (2006). HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. *J. Biol. Chem.* **281**, 35039–35047.
22. Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G., Heinrikson, R. L., Keim, P. *et al.* (1978). The covalent and tertiary structure of bovine liver rhodanese. *Nature*, **273**, 124–129.
23. Miller-Martini, D. M., Chirgwin, J. M. & Horowitz, P. M. (1994). Mutations of noncatalytic sulfhydryl groups influence the stability, folding, and oxidative susceptibility of rhodanese. *J. Biol. Chem.* **269**, 3423–3428.
24. Tanaka, S., Kawata, Y., Otting, G., Dixon, N. E., Matsuzaki, K. & Hoshino, M. (2010). Chaperonin-encapsulation of proteins for NMR. *Biochim. Biophys. Acta*, **1804**, 866–871.
25. Murai, N., Makino, Y. & Yoshida, M. (1996). GroEL locked in a closed conformation by an interdomain cross-link can bind ATP and polypeptide but cannot process further reaction steps. *J. Biol. Chem.* **271**, 28229–28234.
26. Makino, Y., Amada, K., Taguchi, H. & Yoshida, M. (1997). Chaperonin-mediated folding of green fluorescent protein. *J. Biol. Chem.* **272**, 12468–12474.
27. Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B. & Horwich, A. L. (1997). Distinct actions of cis and trans ATP within the double ring of the chaperonin GroEL. *Nature*, **388**, 792–798.
28. Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. (1994). GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell*, **78**, 693–702.
29. Elad, N., Farr, G. W., Clare, D. K., Orlova, E. V., Horwich, A. L. & Saibil, H. R. (2007). Topologies of a substrate protein bound to the chaperonin GroEL. *Mol. Cell*, **26**, 415–426.
30. Motojima, F., Makio, T., Aoki, K., Makino, Y., Kuwajima, K. & Yoshida, M. (2000). Hydrophilic residues at the apical domain of GroEL contribute to GroES binding but attenuate polypeptide binding. *Biochem. Biophys. Res. Commun.* **267**, 842–849.
31. Miller, D. M., Kurzban, G. P., Mendoza, J. A., Chirgwin, J. M., Hardies, S. C. & Horowitz, P. M. (1992). Recombinant bovine rhodanese: purification and comparison with bovine liver rhodanese. *Biochim. Biophys. Acta*, **1121**, 286–292.
32. Nojima, T., Murayama, S., Yoshida, M. & Motojima, F. (2008). Determination of the number of active GroES subunits in the fused heptamer GroES required for interactions with GroEL. *J. Biol. Chem.* **283**, 18385–18392.
33. Watanabe, Y. H. & Yoshida, M. (2004). Trigonal DnaK–DnaJ complex versus free DnaK and DnaJ: heat stress converts the former to the latter, and only the latter can do disaggregation in cooperation with ClpB. *J. Biol. Chem.* **279**, 15723–15727.
34. Taguchi, H., Ueno, T., Tadakuma, H., Yoshida, M. & Funatsu, T. (2001). Single-molecule observation of protein-protein interactions in the chaperonin system. *Nat. Biotechnol.* **19**, 861–865.
35. Ueno, T., Taguchi, H., Tadakuma, H., Yoshida, M. & Funatsu, T. (2004). GroEL mediates protein folding with a two successive timer mechanism. *Mol. Cell*, **14**, 423–434.
36. Koike-Takeshita, A., Shimamura, T., Yokoyama, K., Yoshida, M. & Taguchi, H. (2006). Leu309 plays a critical role in the encapsulation of substrate protein into the internal cavity of GroEL. *J. Biol. Chem.* **281**, 962–967.
37. Yamada, T., Akutsu, N., Miyazaki, K., Kakinuma, K., Yoshida, M. & Oshima, T. (1990). Purification, catalytic properties, and thermal stability of threo-Ds-3-isopropylmalate dehydrogenase coded by leuB gene from an extreme thermophile, *Thermus thermophilus* strain HB8. *J. Biochem.* **108**, 449–456.
38. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J. & Bax, A. (1995). A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR*, **6**, 277–293.