On the Maximum Size of Proteins to Stay and Fold in the Cavity of GroEL underneath GroES*

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GroEL encapsulates non-native protein in a folding cage underneath GroES (cis-cavity). Here we report the maximum size of the non-native protein to stay and fold in the cis-cavity. Using total soluble proteins of Escherichia coli in denatured state as binding substrates and protease resistance as the measure of polypeptide held in the cis-cavity, it was estimated that the cis-cavity can accommodate up to ~57-kDa non-native proteins. To know if a protein with nearly the maximum size can complete folding in the cis-cavity, we made a 54-kDa protein in which green fluorescent protein (GFP) and its blue fluorescent variant were fused tandem. This fusion protein was captured in the cis-cavity, and folding occurred there. Fluorescence resonance energy transfer proved that both GFP and blue fluorescent protein moieties of the same fused protein were able to fold into native structures in the cis-cavity. Consistently, simulated packing of crystal structures shows that two native GFPs just fit in the cis-cavity. A fusion protein of three GFPs (82 kDa) was also attempted, but, as expected, it was not captured in the cis-cavity.

Molecular chaperones are a ubiquitous and abundant group of proteins that play essential roles in folding, assembly, and translocation of other proteins within the cell (reviewed in Ref. 1). One remarkable class of such components is the chaperonins found in bacteria, chloroplasts, mitochondria, archaeabacteria, and eukaryotic cytosol. The best studied of these chaperonins are GroEL and its co-chaperonin GroES from Escherichia coli (reviewed in Refs. 2–4). GroEL is a large cylindrical protein complex comprising two heptameric rings of 57-kDa identical subunits, and these rings are stacked back to back (5). Electron microscopy (6) has indicated that the crystallographically disordered 23-amino acid C-terminal segments of the seven subunits appear to project into the central channel of the cylinder at the level of the ring-ring interface, and hence the central channel of GroEL would function as two cavities, one in each ring. GroES contains seven identical 10-kDa subunits assembled as one heptameric ring (7). The GroEL ring to which GroES is bound is referred to as cis-ring and the opposite ring as trans-ring.

Current understanding of the productive pathway of a GroEL reaction is as follows. (i) Non-native polypeptide binds to GroEL at near the inner rim of the central cavity. (ii) Binding of ATP to the polypeptide-containing ring of GroEL permits the binding of GroES to that ring accompanying the release of polypeptide into an enclosed cage, defined by the GroEL cavity and the dome of GroES, in which folding to the native state can proceed with aggregation being avoided (6, 8–13). (iii) Bound ATP is hydrolyzed (8, 14), and GroES is released upon subsequent ATP binding to the trans-ring of GroEL, permitting native or partially folded proteins to leave GroEL (15).

The mechanism described above imposes a physical limit on the size of a polypeptide whose GroEL-assisted folding is strictly GroES-dependent. Examination of the crystal structure of GroEL/GroES/ADP7 complex suggests that GroES binding fixes a drastic upward and outward shift of the apical GroEL domains, thereby increasing the size of the central cavity and forming a dome-shaped chamber ~85 Å high and ~80 Å wide. This folding cage, termed the cis-cavity, has the volume (175,000 Å3) equal to a globular protein of ~142 kDa, assuming a perfect fit (1.23 Å3/Da) (16), and this must be the theoretical size limit of protein to be contained in the cis-cavity. Actual size limit, however, appears to be far below this limit, since 75-kDa methylmalonyl-CoA mutase was not able to be held in the cis-cavity (9). This may be also the case for 72-kDa phage P22 tailspike protein, since its GroEL-mediated folding did not require GroES (17). These proteins in non-native state can bind to GroEL (in the absence of GroES) or to trans-ring of GroES/GroEL (in the presence of GroES) but cannot fit in the cis-cavity. The question then arises: what is the real size limit of an unfolded polypeptide that can be accommodated within the cis-cavity? To determine this, we have employed the total soluble protein of E. coli as binding substrate of GroEL. It was previously demonstrated that GroEL binds half of the denatured (in vitro) and 10–15% of nascent (in vivo) soluble proteins of E. coli with molecular size ranging from about 10 to 150 kDa (18, 19). However, cis-trans topology of the bound proteins was not clarified in these studies. Using limited proteolysis, we assessed the topology of polypeptides bound to GroEL.

Another question related to (but independent from) the above one is: how large a protein can fold within the cis-cavity? To our knowledge, the protomer of ribulose bisphosphate carboxylase from Rhodospirillum rubrum (55 kDa) is probably the largest molecule for which GroEL-mediated folding shows strict GroES dependence, an indication of folding in the cis-cavity (20). However, ribulose bisphosphate carboxylase is a homodimer enzyme, and its catalytic activity, the measure of proper folding, can only be assayed after the release from GroEL to the bulk medium. As a consequence, it is not certain if the protomer of this enzyme completes the folding in the cis-cavity or after the escape from the cis-cavity. Completion of folding in the cis-cavity has been demonstrated only for three relatively small proteins, namely 33-kDa rhodanese (13), 27-
kDa green fluorescent protein (GFP)

Reagents and Proteins—ATP, hexokinase, proteins K, DNAse I, and RNase A were purchased from Roche Molecular Biochemicals. AMP-PNP, bovine serum albumin, and trypsin were from Sigma. ADP was from Biochemlab. N-(4-2-aminoethyl)benzenesulfonfonyl fluoride hydrochloride was from Wako (Tokyo, Japan). Alkaline phosphatase-conjugated streptavidin and anti-GFP antibodies were obtained from Vector Laboratories and CLONTECH, respectively. GroEL and GroES were purified as follows. E. coli strain BL21(DE3), bearing GroEL expression plasmid pET-EL, was cultured, collected, sonicated, and centrifuged as described previously. The plasmid pET-EL, was cultured, collected, sonicated, and centrifuged (20,000 × g for 20 min). Supernatant fraction was applied on a Butyl-Toyopearl column (Tosoh) equilibrated with 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM (4-2-aminoethyl)-benzenesulfonfonyl fluoride hydrochloride, sonicated, and centrifuged, (20,000 × g for 20 min). Supernatant fraction was fractionated (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% methanol, and 10% saturated ammonium sulfate, and the resulting fractions were collected. GroEL was eluted at a volume with a 10–0% saturated ammonium sulfate linear gradient. GroEL fractions were collected, dialyzed against 20 mM Na-P (pH 7.5), and stored at –80 °C until use. E. coli cells BL21(DE3), bearing GroES expression plasmid pET-ES, was cultured, collected, sonicated, and centrifuged as above. Supernatant fraction was applied on a Butyl-Toyopearl column equilibrated with 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 20% saturated ammonium sulfate, and the column was washed with 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT, 20% saturated ammonium sulfate, and 10% methanol. GroES was then eluted with a 20–0% saturated ammonium sulfate linear gradient in the buffer without methanol. GroES fractions were collected, dialyzed against 20 mM Na-P (pH 7.5), and stored at –80 °C until use. Construction of Plasmids for GFPs—All GFP mutants were generated by site-directed mutagenesis using the Kunkel method (22), and single-stranded DNA of plasmid was obtained by infecting E. coli CJ236 cells with helper phage M13KO7 (Amersham Pharmacia Biotech). A Nhel-EcoRI fragment containing the gfp gene was isolated from wild-type GFP-expressing plasmid pETU658 (a kind gift from Dr. Martin Challie; Ref. 23), and inserted into pET21c (Novagen) in Nhel-EcoRI sites (pET-wtGFP). Using single-stranded DNA of pET-wtGFP as a template, plasmid pET-GFPPer was generated by site-directed mutagenesis. The plasmid pET-GFPPer encodes a mutant GFP (F99S/M153T/V163A), that shows improved folding efficiency (24). The peak wavelengths of the excitation and fluorescence spectra of this GFP mutant is identical to those of the wild-type GFP. Next, using single-stranded DNA of pET-GFPPer as a template, pET-GFPPer and pET-BFP-GFPPer were generated. pET-GFPPer encodes a GFP mutant that has two more amino acid substitutions, F64L/S65C, that allow the protein to fold in the cis-cavity. The mutant GFP was digested with XhoI, and both termini of the fragment containing gfp gene were ligated together. This procedure generated the plasmid pET-GFPPer-His, which expresses C-terminal histidine tagged GFP. From the pET-GFPPer-His, Nhel-Nhel-compatible XhoI fragment was isolated and ligated into Nhel site of pET-GFPPer-His. The resultant plasmid, pET-BFGFPPer-His, encoded a fusion protein of GFP and BFP with His tag at the C terminus. Amino acid sequence of the protein expressed from this plasmid was deduced as: MA-{BFP(S2... F64L/Y66H/F99S/Y145F/M153T/V163A... G232)}-SGGRLEGSGS-(GFP-S2... F64L/65C/F99S/M153T/V163A... G232)}-SGGRLEHHHHHHH. Trypsin cleaves the peptide bonds after Arg in the linker region and in the C-terminal tail. The plasmid pET-BFP-GFPPer-His, which encoded C-terminal His-tagged BFP, was produced by inserting NspV-ResIII fragment from pET-BFP-GFPPer into the similarly cut plasmid pET-GFPPer-His. For simplicity, these His-tagged mutant proteins are denoted by GFP and BFP and GFP in this paper.

Purification of BFP-GFP—E. coli BL21(DE3) cells were transformed with pET-BFGFPPer-His, cultured at 37 °C to an OD600 of ~0.5, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside for overnight (15 ± 5°C). Cells were harvested, suspended and sonicated in 25 mM Na-P, (pH 7.5) which contained a protease inhibitor mixture, Complete (Roche Molecular Biochemicals), and 500 mM NaCl. The disrupted cells were centrifuged (20,000 × g for 20 min), and the supernatant was applied on a Ni-NTA-agarose column (Qiagen) equilibrated with 25 mM Na-P, (pH 7.5), 500 mM NaCl. With a 0–500 mM imidazole linear gradient, BFP-GFP was eluted at around 50 mM imidazole. The BFP-GFP fraction was next applied on a Butyl-Toyopearl column equilibrated with 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT, 20% saturated ammonium sulfate, and eluted with a 20–0% saturated ammonium sulfate gradient. Fractions containing BFP-GFP were collected, dialyzed against 25 mM Tris-Cl (pH 7.5), and stored at ~80 °C until use. BFP and GFP were individually purified through the similar procedures above described from E. coli BL21(DE3) cells transformed with pET-BFP-GFPPer-His or pET-GFPPer-His, respectively.

Proteinase Sensitivity of GroEL-bound Polypeptides—All reactions were carried out at 25 °C. Biotinylated E. coli soluble proteins in 8 M urea (125 μM) was diluted 100-fold into the dilution buffer (50 mM M-KCl, 100 mM Na2SO4, 1 mM Mg(CH3COO), and 5 mM DTT), which contained 2.5 μM GroEL and 5.0 μM GroES. The solution was incubated for 1 min, and nucleotide, 50 μM ATP (1.43 mol/mol GroEL protomer) or 1 mM AMP-PNP (final concentrations), was added. In the case of ADP, the dilution buffer containing 2.5 μM GroEL, hexokinase (0.45 units), 20 mM glucose, and 1 mM ADP, was preincubated for 5 min to remove contaminating ATP. To this solution, biotinylated E. coli soluble proteins were added and incubated 100-fold and, after 1 min, 2.5 μM GroES was added. The mixtures containing ATP, AMP-PNP, or ADP thus prepared were incubated for 5 min, and then proteinase K was added to 10 μg/ml. After 30 min, reaction was stopped by addition of 5 mM (4-2-aminoethyl)-benzenesulfonfonyl fluoride hydrochloride. The reaction mixture was centrifuged (17,000 × g for 5 min at 25 °C), and an aliquot (80 μl) was applied to a gel filtration HPLC column (G3000SW TSK, Tosoh). The column was equilibrated and eluted with 50 mM HEPES-NaOH (pH 7.0), 10 mM Mg(CH3COO), 5 mM KCH3COO, 100 mM Na2SO4, at a flow rate of 0.5 ml/min. The (GroES/polypeptide/GroEL complex was isolated, and was analyzed by SDS-PAGE followed by blotting to polyvinylidene difluoride membrane. Biotinylated proteins were visualized by alkaline phosphatase-conjugated streptavidin. In the experiments with BFP-GFP, acid denatured BFP-GFP solution (25 μM) in 12.5 mM HCl, 1 mM DTT, 8 mM Tris-Cl (pH 7.5) (final pH 1.5) was diluted 20-fold into the dilution buffer containing 2.5 μM GroEL and 5.0 μM GroES and, after 1 min, 50 μM ATP was added. The following procedures were the same as the above, except that anti-GFP antibodies were used to detect BFP-GFP. GroEL reacted neither with streptavidin nor with anti-GFP antibodies.

BFP-GFP Folding Analysis—BFP-GFP folding reactions were carried out at 25 °C. Acid-denatured BFP-GFP solution (25 μM) was diluted 25-fold into the dilution buffer containing 2.0 μM GroEL, 4.0 μM GroES, and 0.5 mg/ml bovine serum albumin. The solution was centrifuged for 1 min, and 40 μM ATP was added to start GroEL-mediated folding. After a 20-min incubation, the solution was subjected to a brief...
The total soluble protein of *E. coli* was denatured, biotinylated and used as binding substrate (Total labeled proteins). The polypeptide/GroEL complex and GroES/polypeptide/GroEL complex were isolated and analyzed with SDS-PAGE. Polypeptides were visualized by alkaline phosphatase-streptavidin. Lanes 1 and 2, polypeptide/GroEL complex; lanes 3 and 4, GroES/polypeptide/GroEL complex formed in the presence of nearly stoichiometric ATP; lanes 5 and 6, GroES/polypeptide/GroEL complex formed in the presence of ADP; lanes 7 and 8, GroES/polypeptide/GroEL complex formed in the presence of AMP-PNP. In lanes 2, 4, 6, and 8, complexes were treated with protease K. Details of the experiment are described under “Materials and Methods.” Because of the large amount of GroEL employed in the reactions, polypeptides around 57 kDa (equal to the GroEL protomer) were compressed downward.

centrifugation (17,000 × g for 5 min at 25 °C) and an aliquot (50 μl) was injected into a gel filtration HPLC column (G3000SWXL, Tosoh). Elution was monitored with an on-line spectrophotometer and an on-line fluorometer (FS-8010, Tosoh).

**Trypsin Treatment of BFP-GFP, BFP, and GFP—Cleavage of BFP-GFP, BFP, and GFP by trypsin was carried out at 25 °C under two conditions.** For SDS-PAGE, 10 μg of BFP-GFP, BFP, or GFP was incubated in a 50-μl solution containing 25 mM Tris-Cl (pH 7.5), 20 mM CaCl₂, and 6 μg of trypsin. At the time indicated, an aliquot (10 μl) was taken out and 4-2 (aminoethylene)benzenesulfonyl fluoride hydrochloride (5 mM, final concentration) was added to stop the reaction. Aliquots treated in this manner were analyzed with SDS-PAGE, and proteins were stained with Coomassie Blue R-250. For the spectroscopic analysis, 20 μg of BFP-GFP was incubated in a 1.2-mL solution containing 25 mM Tris-Cl (pH 7.5), 20 mM CaCl₂, and 30 μg of trypsin, and fluorescence spectra by excitation light at 380 nm were measured with a fluorometer (FP-777, Jasco) at indicated time intervals.

**Preparation of GFP Trimer**—The sites of XhoI and XhoI were introduced into pET-GFP at the 3’-terminus of the gfp gene and a resultant plasmid, pET-GFPPer-c, was digested with XhoI. The fragment that contained the gfp gene was ligated both ends together to attach 6×His tag at the C terminus of GFP and was named pET-GFP(His). Two gfp genes containing NheI-XhoI fragment obtained from pET-GFPPer-c were ligated into the NheI site of pET-GFP(His), and a plasmid, pET-3GFP(His), encoding a GFP trimer with a C-terminal 6×His tag was produced. The linker sequence connecting each GFP moiety was Arg-Gly-Leu. GFP trimers were expressed in *E. coli* BL21(DE3) transformed with pET-3GFP(His) as inclusion bodies. Inclusion bodies were washed with 1% Triton X-100, solubilized in 100 mM Na-P₃ (pH 7.5), 6 mM guanidine-HCl, 5 mM 2-mercaptoethanol, and applied on a Ni-NTA-agarose column equilibrated with the same buffer. The column was washed with 100 mM Na-P₃ (pH 7.5), 8 mM urea, 5 mM 2-mercaptoethanol and was eluted with a linear gradient from 100 mM Na-P₃ (pH 7.5) to 100 mM sodium citrate (pH 4.0) in 8 mM urea, and 5 mM 2-mercaptoethanol. Fraction of GFP trimer was dialyzed to 25 mM Tris-HCl (pH 7.5), 8 mM urea, and stored at −80 °C until use. This GFP trimer preparation was not completely homogeneous but pure enough for the following experiment. The protease sensitivity experiment was carried out in the same manner as described in the experiment with BFP-GFP, except that 20 μM denatured GFP trimer in 25 mM Tris-HCl (pH 7.5), 8 mM urea, 12.5 mM HCl, 1 mM DTT (final pH 3.0) was diluted 50-fold into the dilution buffer containing 1.0 μM GroEL and 2.0 μM GroES. Next, 20 μM ATP and then 4 μg/ml protease K were added as indicated.

**RESULTS**

**Size of the Proteins in the cis-Cavity**—Polypeptides bound to GroEL are highly susceptible to proteolysis (8, 31, 32). However, when GroEL binds polypeptide first and GroES and ATP (or ADP) next, undigested polypeptides remain (9–11). This is because polypeptide attached at the cis-ring of GroEL becomes sequestered in the cis-cavity upon binding of GroES and is protected from protease. Taking advantage of this protection, we analyzed the size of the proteins that were able to be accommodated in the cis-cavity (Fig. 1). Total soluble protein of *E. coli* was urea-denatured, biotinylated, and used as binding substrate that was the mixture of proteins of various size (Fig. 1, Total labeled protein). The mixture was diluted into the buffer containing GroEL and GroES, and allowed to form polypeptide/GroEL complex. Then, for indicated cases, ATP, ADP, or AMP-PNP was added to form GroES/polypeptide/GroEL complex. Note that the amount of ATP was slightly over...
Fig. 3. Folding of the BFP-GFP in the cis-cavity. A, protection of the BFP-GFP from proteinase K digestion. Denatured BFP-GFP was diluted into the buffer containing GroEL and GroES in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of nearly stoichiometric ATP and treated with proteinase K (lanes 2 and 4). The (GroES)/BFP-GFP/GroEL complex was isolated, electrophoresed, blotted, and visualized with anti-GFP antibodies. Details of the experiment are described under “Materials and Methods.”

B, gel filtration HPLC elution of the GroES/BFP-GFP/GroEL complex formed in the presence of ATP. The BFP-GFP was diluted into the buffer containing GroEL and GroES in the presence of nearly stoichiometric ATP. After a 20-min incubation, the solution was applied to a G3000SWxl column. Elution was monitored with fluorescence at 510 nm exciting by 380 nm, and UV absorbance at 280 nm. Scales of ordinates are arbitrary. The absorbance peaks at 17.5 and 15.5 min were bovine serum albumin and an unknown contaminating protein in it, respectively. C, fluorescence resonance energy transfer of the BFP-GFP held in the cis-cavity. Wavelength of excitation light was 380 nm. The GroES/BFP-GFP/GroEL complex formed in the presence of nearly stoichiometric ATP was isolated, electrophoresed, blotted, and visualized with anti-GFP antibodies. Polypeptides of various size ranging from about 8 to 110 kDa were found to be associated with the GroEL complex for all samples (Fig. 1, lanes 1, 3, 5, and 7). By treatment with proteinase K prior to HPLC, polypeptides associated with the complex that had been formed in the absence of nucleotide disappeared completely (Fig. 1, lane 2). However, in the cases of the complexes that had been formed in the presence of nucleotides, significant amount of polypeptides were protected from digestion even though majority of bound polypeptides disappeared (Fig. 1, lanes 4, 6, and 8). Polypeptides lost by the protease treatment may represent those bound to trans-ring of GroEL and possibly those improperly bound to cis-ring. The protease-resistant polypeptides represent a fraction of polypeptides held in the cis-cavity; noticeably, they are all smaller than the 57-kDa GroEL subunit. Therefore, we have concluded that the maximum molecular size to be contained in the cis-cavity is ~57 kDa.

BFP-GFP—The above experiments did not reveal whether folding in the cis-cavity would be possible for the proteins with nearly maximum size. To determine this, we made a 54 kDa BFP-GFP in which blue fluorescent variant of GFP (BFP) and green fluorescent GFP were fused in a single polypeptide. Both BFP moiety and GFP moiety in the BFP-GFP purified from expressing E. coli cells were proved to have native structures from two criteria. As in the case of wild-type GFP, individual monomers of BFP and GFP were resistant to trypsin digestion (Fig. 2A, left and center lanes). Shift of the original protein band to a slightly lower band with increasing incubation time reflected the cleavage of the C-terminal tail connecting His tag. When the BFP-GFP was treated with trypsin, the linker regions were cleaved by trypsin and the BFP-GFP band disappeared in parallel with appearance of stable bands of GFP and BFP that were no longer digested except for cleavage of the GFP C-terminal tail (Fig. 2A, right lanes). Another support for native structures of BFP and GFP moieties in the BFP-GFP was the fluorescence resonance energy transfer (FRET). When the BFP moiety of the BFP-GFP was excited by 380 nm light, the stoichiometric amount to the GroEL protomers (1.43 mol of ATP/mol of protomer) so that most ATP would be exhausted before the release of polypeptides from GroEL, which was also an ATP-requiring process. The complex was isolated with gel filtration HPLC, electrophoresed, blotted onto a membrane, and visualized by using biotin tag. Polypeptides of various size ranging from about 8 to 110 kDa were found to be associated with the GroEL complex for all samples (Fig. 1, lanes 1, 3, 5, and 7). By treatment with proteinase K prior to HPLC, polypeptides associated with the complex that had been formed in the absence of nucleotide disappeared completely (Fig. 1, lane 2). However, in the cases of the complexes that had been formed in the presence of nucleotides, significant amount of polypeptides were protected from digestion even though majority of bound polypeptides disappeared (Fig. 1, lanes 4, 6, and 8). Polypeptides lost by the protease treatment may represent those bound to trans-ring of GroEL and possibly those improperly bound to cis-ring. The protease-resistant polypeptides represent a fraction of polypeptides held in the cis-cavity; noticeably, they are all smaller than the 57-kDa GroEL subunit. Therefore, we have concluded that the maximum molecular size to be contained in the cis-cavity is ~57 kDa.

In this paper, we use the term GFP often as a folding-efficient GFP mutant (see “Materials and Methods”) in the sentences where there is no possible confusion.
excitation of the GFP moiety was induced and eventually the BFP-GFP emitted green fluorescence at 520 nm (Fig. 2B, trace 1). This efficient FRET can occur only when BFP and GFP moieties are located very closely, in the same protein in this case. In fact, as BFP and GFP moieties in the same BFP-GFP complex emitted green fluorescence of GFP by 380 nm excitation light, a suitable excitation light for BFP (Fig. 3B). This fluorescence was really emitted from the BFP-GFP held in the cis-cavity of the GroES/GroEL complex, since addition of EDTA, which induced the release of GroES from GroEL, resulted in disappearance of fluorescence from the GroEL peak fraction (data not shown). Folded, free BFP-GFP was also eluted at 19 min, which were probably BFP-GFP molecules released from the cis-cavity and/or trans-ring of GroEL. Because native BFP-GFP by itself does not bind to GroEL (data not shown), native BFP-GFP associated with the GroES/GroEL complex should be a product of folding in the cis-cavity.

To analyze contribution of FRET to the fluorescence at the GroEL peak fraction of HPLC, the GroES/BFP-GFP/GroEL complex was isolated, and its fluorescence spectrum was measured (Fig. 3C). In principle, the spectrum would have been the sum of contributions of fully folded BFP-GFP, in which both BFP and GFP moieties completed folding, and of half-folded BFP-GFP, in which only one of moieties completed folding. We estimated the relative contribution of the fully folded BFP-GFP in the observed spectrum on the assumption that the populations of two kinds of half-folded BFP-GFP (“BFP-only” molecule and “GFP-only” molecule) were the same. Based on this assumption and using the spectra in Fig. 2B as references, one can calculate that the spectrum in Fig. 3C is generated by a mixture of ~60% fully folded BFP-GFP and ~40% half-folded BFP-GFP. The spectrum of 100% fully folded BFP-GFP, as well as the spectrum of 100% half-folded BFP-GFP (=0% fully folded BFP-GFP), at the same concentration of BFP-GFP are also calculated and shown in Fig. 3B (100% and 0%). The assumption of equal population of BFP-only molecules and GFP-only molecules may not be far from the case, since inherent folding ability of BFP and GFP moieties in BFP-GFP are similar as described in the above paragraph. Furthermore, even without this assumption, occurring of FRET in the GroES/BFP-GFP/GroEL complex is evident. If whole fluorescence at 510 nm by 380 nm excitation light of the observed spectrum in Fig. 3C has been emitted solely by GFP-only molecules but not derived from FRET, we can predict the amount of GFP-only molecules and hence the intensity of the 510 nm fluorescence excited by 480 nm light, an optimum excitation light for GFP. However, the observed intensity at 510 nm excited by 480 nm was much smaller than the predicted one (data not shown) and could not be explained without FRET. Altogether, we conclude that significant fraction of BFP-GFP molecules within the cis-cavity achieve complete folding; both BFP and GFP moieties reach the native structures.

**Protease Digestion of GroEL-bound GFP Trimer**—We also made an 82-kDa protein consisting of three GFPs. The denatured trimer could bind to GroEL in the absence and presence of ATP (Fig. 4, lanes 1 and 3), but, as expected, bound GFP trimer was completely digested by protease K (Fig. 4, lanes 2 and 4). ATP did not protect bound GFP trimer from digestion. The GFP trimer is too large to be accommodated in the cis-cavity.

**DISCUSSION**

Although the crystal structure of GroES/GroEL complex imposes ~142 kDa as a physical size limit of protein to be accommodated in the cis-cavity (16), the actual size limit has not been determined by experiments. Here, we have shown that up to ~57-kDa protein, but no larger proteins, can be accommodated in the cis-cavity and that at least a 54-kDa protein can finish folding there. Thus, actual size limit is less than half of the maximum physical size limit. Folding intermediates have more expanded volume, and random thermal motion of polypeptide chain of the intermediate should be allowed to find the proper place(s) where they can be accommodated in the cis-cavity.
interactions between segments distant in the primary sequence. Also, because interactions with surrounding water molecules are essential to stabilize native structure of the soluble proteins, the cis-cavity must contain many water molecules for the folding intermediate to reach the native structure. Entropic burden to pack a large extended polypeptide chain into a restricted space may be another factor limiting the size.

The shapes of folded, native proteins are not always exactly globular, and the fit of the shape of native protein with that of the cis-cavity may also be important. The 54-kDa protein we tested for folding is a fused dimer of GFP, and a monomer GFP is a 27-kDa protein with the shape of a cylinder, which has a diameter of ~30 Å and length of ~40 Å (33, 34). Using the crystal structures of GFP and GroES/GroEL complex, we tried to pack the GFP dimer into the cis-cavity and found that the dimer can just fit in the cis-cavity without steric collision (Fig. 5). Three GFP molecules cannot fit in by any means. Interestingly, a 56-kDa T4 phage head protein (gp23) may not be contained in the cis-cavity underneath GroES and the phage encodes its own derivative of GroES, gp31, which can form a larger cis-cavity to accommodate gp23 (35).

Now that ~57 kDa is the cut-off size of the cis-cavity, how do the larger proteins fold in vivo? Pulse-labeling experiment of E. coli cells showed that proteins larger than 57 kDa actually bound to GroEL, even if the population was small and commitment of GroES was not clear (19). Furthermore, the bound large proteins were retained to GroEL in a more stable manner during the chase period than the proteins of molecular size 25–55 kDa. Implication of this observation remains open. It was recently reported that hsp60 (GroEL homologue)-mediated in vivo folding of a 87-kDa mitochondrial aconitase in yeast was dependent on hsp10 (GroES homologue) (36). The question of whether GroEL (and GroES) indeed assists the folding of large proteins in vivo has not been answered and should gain more attention.

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