Encapsulation of an 86-kDa Assembly Intermediate inside the Cavities of GroEL and Its Single-ring Variant SR1 by GroES*

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We described previously that during the assembly of the \(\alpha_2\beta_2\) heterotetramer of human mitochondrial branched-chain \(\alpha\)-ketoacid dehydrogenase (BCKD), chaperonins GroEL/GroES interact with the kinetically trapped heterodimeric \((\alpha\beta)\) intermediate to facilitate conversion of the latter to the native BCKD heterotrimer. Here, we show that the 86-kDa heterodimeric intermediate possesses a native-like conformation as judged by its binding to a fluorescent probe 1-anilino-8-naphthalenesulfonate. This large heterodimeric intermediate is accommodated as an entity inside cavities of GroEL and its single-ring variant SR1 and is encapsulated by GroES as indicated by the resistance of the GroES complex isolated as a stable single species by gel filtration in the presence of Mg-ATP. In contrast, an unfolded BCKD fusion protein of similar size, which also resides in the GroEL or SR1 cavity, is too large to be trapped by GroES. The cis-capping mechanism is consistent with the high level of BCKD activity recovered with the GroEL-\(\alpha\beta\) complex, GroES, and Mg-ATP. The 86-kDa native-like heterodimeric intermediate in the BCKD assembly pathway represents the largest protein substrate known to fit inside the GroEL cis cavity underneath GroES, which significantly exceeds the current size limit of 57 kDa established for unfolded proteins.

Chaperonins GroEL/GroES, which are homologues of mitochondrial Hsp60/10, respectively, have been shown to promote proper folding and assembly of a variety of proteins (for review see Refs. 1 and 2). GroEL is a double-ring complex with two heptameric rings, consisting of identical 57-kDa subunits, which are stacked back to back. The efficient folding for most proteins has been shown to occur inside the cavity of the cis ring that houses the unfolded or partially folded protein, following encapsulation or capping of the cis ring by dome-shaped heptameric GroES in the presence of Mg-ATP (3). The subsequent binding of Mg-ATP to the unoccupied or trans ring of GroEL results in the collapse of the cis ring assembly with the concomitant release of GroES and partially or completely folded protein from the cis cavity. It has been generally accepted that only proteins of 55–57 kDa or smaller in size can fit inside the cavity encapsulated by GroES, as shown by in vivo and in vitro (4, 5) and in vivo (6) studies. For certain proteins larger than this size, for example, 75-kDa methylmalonyl-CoA mutase (3) and 72-kDa phage P22 tailspike protein (7) that are capable of binding to GroEL, chaperonin-assisted folding is independent of GroES. It was shown recently, however, that both chaperonins GroEL/GroES are required for the productive folding of an 86-kDa maltose-binding protein fusion (8) and 82-kDa mitochondrial aconitase (9). Interestingly, for this group of large proteins, productive folding is achieved through binding of GroES to the trans ring of GroEL. GroEL was also shown to trap heat-induced inactive 98-kDa citrate synthase homodimers (10) and thermally induced partially active 66-kDa rhodanese dimers (11). In the presence of GroES and Mg-ATP, the trapped dimers of citrate synthase or rhodanese are released from GroEL in the monomeric form. It is not clear whether GroES binds in cis or in trans to the complex formed between GroEL and these relatively large folded intermediates.

Our laboratory is interested in the potential role of chaperonins in promoting the assembly of mitochondrial macromolecular multi-enzyme complexes. We have shown that chaperonins GroEL/GroES are indispensable for both folding and assembly of the \(\alpha_2\beta_2\) heterotetramer of branched-chain \(\alpha\)-ketoacid dehydrogenase (BCKD), a component of the \(4 \times 10^6\)-Da human mitochondrial BCKD complex, both in Escherichia coli (12) and in vitro (13). GroEL binds to an ensemble of 86-kDa \(\alpha\beta\) heterodimeric intermediates, which are kinetically trapped during assembly, to produce a stable GroEL-\(\alpha\beta\) complex. In the presence of GroES and Mg-ATP, the heterodimeric intermediate undergoes multiple rounds of dissociation and reassociation to facilitate the conversion of the heterodimeric intermediate to the native BCKD heterotrimer (14, 15). Our data have established the central role of chaperonin GroEL/GroES in promoting oligomeric protein assembly through iterative annealing of non-productive assembly intermediate.

Because of the large size associated with the \(\alpha\beta\) assembly intermediate of BCKD, a question arises as to whether the productive folding of the GroEL-\(\alpha\beta\) complex proceeds through the cis or trans capping of the GroEL cavity by GroES. We have shown previously that the GroEL-\(\alpha\beta\) complex is resistant to protease digestion in the presence of GroES and Mg-ADP, suggesting the enclosure of the 86-kDa heterodimer inside the GroEL cis cavity by GroES (15). However, the interpretation of these data was complicated by the presence of the trans ring in GroEL. In the present study, we revisited this issue by investigating the encapsulation of the heterodimer inside GroEL along with its single-ring variant SR1. We showed that the 86-kDa heterodimeric intermediate inside the cavities of both

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GroEL and SR1 was encapsulated by GroES. In contrast, an unfolded fusion protein of similar size, which also resides in the GroEL or SR1 cavity, is too large to be capped by GroES. The 86-kDa heterodimeric intermediate represents the largest protein substrate ever known to fit inside the GroEL cis cavity underneath GroES. This finding indicates that for assembly intermediates with compact native-like conformations, the size limit for cis folding is significantly larger than 57 kDa established for unfolded proteins (4–6).

EXPERIMENTAL PROCEDURES

Materials—Bacterial chaperonins GroEL/GroES and recombinant human BCKD were prepared as described previously (13). 1-Anilino-8-naphthalenesulfonate (ANS), trypsin (type III, from bovine pancreas), trypsin-chymotrypsin inhibitor (from soybean), Reactive Red 120-agarose (type 3000-CL), and α-lactalbumin (type III, calcium-depleted, from bovine milk) were obtained from Sigma. Bovine lactoferrin was purchased from ICN Biomedicals, Inc. (Aurora, OH). A pET vector for the expression of single-ring GroEL variant SR1 was a generous gift from Dr. Arthur Horwich.

Expression and Purification of SR1—The expression plasmid for SR1 was transformed into BL21 (DE3) cells. Cells were grown in the LB medium at 37 °C to an OD600 of 0.6 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. The SR1 protein was purified at 4 °C using a previously described protocol with modifications (16). Briefly, cells from 4-liter culture were collected and resuspended in 150 ml of buffer containing 20 mM Tris, pH 7.4, 50 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mg/ml lysozyme. After cells were broken open, the lysate was fractionated by centrifugation. Following centrifugation, the protein pellet was dissolved in Buffer A, and solubilized proteins were fractionated on an FPLC Superdex 200 gel filtration column equilibrated in the same buffer. At this stage, the SR1 protein was >98% pure as judged by SDS-PAGE. To remove minor contaminants, it was dialyzed against 20 mM Tris, pH 7.4, and 5 mM MgCl2, and purified further on a Reactive Red 120-Agarose (type 3000-CL) dye column equilibrated in the same buffer (17).

Engineering and Expression of BCKD α-11-β Fusion Protein—For the construction of an expression plasmid for the α-11-β fusion protein, three fragments from the phHisTE1 vector (18) were produced: a 45-bp NcoI/Xhol fragment containing the Hisα tag and the tobacco etch-virus protease cleavage site, a 751-bp XhoI/SmaI fragment encoding a 5′ terminal segment of the α subunit, and a 1202-bp PstI/EcoRI fragment encoding a Cterminal segment of the β subunit. The linker region of 11 amino acids (GSEALEAAERS) between the α and β subunit was derived from the sequence connecting the α and β domains of tryptophan synthase (19).

The nucleotide sequence encoding this linker was 5′-GGATCCGAAGATCAGTTAGAAAGCGCCGCTAGACTC-3′, which contained a BamHI site at the 5′ end and a BgIII site at the 3′ terminus. The BCKD-α cDNA with the linker immediately downstream of the 3′ cDNA end was amplified, followed by digestion of the PCR product to produce a SmallI/BamHI fragment. The BCKD-β cDNA with the linker attached to the 5′ cDNA end was also amplified, and digestion of this PCR product resulted in a PstI/BamHI fragment. The SmallI/BamHI and PstI/BamHI fragments, along with the above three fragments derived from the phHisTE1 plasmid, were ligated into the pTrcHisB expression vector (Invitrogen) pre-digested with NcoI and EcoRI. The resultant plasmid phHisTE-α-11-β and the pGroESL plasmid overexpressing GroEL and GroESL were transformed into the BL21 cells. Transformed cells were grown in the LB medium at 37 °C until A600 was 0.75 was reached. Expression of the BCKD fusion protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, followed by an overnight growth at 37 °C. The Hisα-tagged BCKD α-11-β fusion protein was isolated from the cell lysate with nickel-nitrilotriacetic acid-agarose as described previously (18).

Preparation of GroEL-Protein and SR1-Protein Complexes—KSCN-induced αβ heterodimers were produced by incubating Hisα-tagged BCKD (115 μM, αβ heterotetramer) with 400 μM KSCN in Buffer B (50 mM potassium phosphate, pH 7.5, 250 mM KCl) containing 2 mM dithiothreitol for 45 min at 23 °C (14). The incubation mixture was diluted 2-fold into Buffer B containing 8 μM GroEL or SR1. Following incubation for 4 h at 23 °C, the complex formed was purified on an FPLC Superdex 200 gel filtration column. Fractions containing the GroEL-αβ or the SR1-αβ complex were collected and concentrated in a Millipore (Bedford, MA) Ultrafree-15 filter device with a 30-kDa cut-off membrane.

GroEL-α-11-β and SR1-α-11-β complexes were prepared by diluting the αβ fusion protein in the presence of 0.5 M KSCN to 13 mM Mg-ADP, pre-subjected to limited digestion with 20 μg/ml trypsin at 23 °C for 10 min. The reaction was terminated by adding trypsin-chymotrypsin inhibitor to a final concentration of 27 μg/ml. Samples were analyzed by SDS-PAGE and Western blotting using a combination of anti-α subunit and anti-β subunit antibodies as a probe.

Fluorescence Assays of BCKD and BCKD Fusion Proteins with ANS—The urea-denatured α-11-β fusion protein was generated by incubation with 8 M urea at 23 °C for 1 h. Native BCKD, the KSCN-induced heterodimer, or the urea-denatured α-11-β fusion protein at 0.1 mg/ml was mixed with 50 μM ANS in Buffer C. Emission spectra were recorded from 400 to 550 nm with the excitation wavelength at 365 nm on a PerkinElmer Life Sciences luminescence spectrometer LS 50B. Each spectrum was an average of three consecutive scans and was corrected for contributions from the buffer solution.

Co-purification of GroES with SR1-αβ or SR1-β Complexes by HPLC and Quantification of Protein Components by Densitometry—The SR1-β complex was prepared as described previously except that SR1 was used instead of GroEL (13). The SR1-αβ or SR1-β complex (60 μg) was incubated with 100 μg of GroES in Buffer C containing 5 mM Mg-ATP for 5 min at 23 °C. The mixture was fractionated on an HPLC G3000SWxl gel filtration column in the same buffer containing 0.1 mM Mg-ATP. As a control, the SR1-β complex was incubated with GroES in the absence of Mg-ATP and separated on HPLC with Mg-ATP omitted from the column buffer. 5 min after the sample injection, fractions were collected every 20 s and analyzed by SDS-PAGE. To determine the stoichiometry of protein components in the SR1-αβ-GroES or the SR1-β-GroES complex, different amounts of SR1, BCKD, or GroES were run on the same gel. After staining with Coomassie Blue, gels were scanned on a Molecular Dynamics densitometer (model 300A) and analyzed by ImageQuant. Standard curves for SR1, BCKD, and GroES were generated by plotting the known amount of the protein against the intensity of the band on the same gel. The amount of each protein component in the SR1-αβ (or α-β)-GroES complex was calculated using the standard curve for each protein.

GroEL-GroES-mediated Recovery of BCKD Activity—The renaturation of urea-denatured BCKD and α-11-β fusion protein was carried out as described previously (13). BCKD activity was also recovered by incubating 0.8 μM of the GroEL-αβ or the GroEL-α-11-β complex with 2 μM GroES and 10 mM Mg-ATP as described previously (14).

RESULTS

Expression and Characterization of Large Target Proteins—The Hisα-tagged αβ heterodimeric intermediate in the BCKD assembly pathway was produced by incubating the N-terminally Hisα-tagged BCKD αβ heterotetramer in 400 mM KSCN for 45 min at 23 °C, followed by a 2-fold dilution. The size of the resultant αβ heterodimer was 86 kDa, as determined by FPLC gel filtration and dynamic light scattering in the presence of 200 mM KSCN (14). A second target protein, the Hisα-tagged α-11-β fusion, was generated by fusing the N-terminally Hisα-tagged α subunit to the β subunit through an 11-residue linker derived from E. coli tryptophan synthase (19). The α-11-β fusion migrated as a 170-kDa species in FPLC gel filtration, similar to the size of wild-type BCKD heterotetramer. The α-11-β fusion shows Km values of 0.5 μM for thiamin pyrophosphate and 62 μM for substrate α-ketoisovalerate, essentially indistinguishable from those obtained with the wild-type BCKD (Table I). The kcat value for the α-11-β fusion is 4.0 s−1, which is approximately one-half that obtained with the wild-type BCKD. The size of the 8 M urea-denatured α-11-β fusion...
was determined by sedimentation equilibrium centrifugation (data not shown). The data were fitted to a single species model with a size of 86,671 Da for the unfolded α-11-β fusion polypeptide. The above data, taken together, indicate that the native α-11-β exists as a homodimeric protein.

Conformations of the target proteins were characterized by fluorometry using the ANS as a hydrophobic probe. Native and urea-denatured proteins were incubated with 50 μM ANS. The reaction mixtures were excited at 365 nm, and fluorescence emission measured as a function of wavelength (Fig. 1). ANS bound to the αβ assembly intermediate or the αβ native BCKD exhibits similar spectra with a maximum at 473 nm. The data indicate that the heterodimeric assembly intermediates possesses a folded structure similar to native BCKD. In contrast, the urea-denatured α-11-β fusion with the unfolded conformation fail to bind to ANS, as indicated by the absence of fluorescence emission after subtracting emission from the unbound ANS.

Capping of the 86-kDa Native-like Heterodimeric Intermediate inside GroEL and SR1 Cavities by GroES—Double-ring GroEL or its single-ring variant SR1 was incubated with a molar excess of KSCN-induced αβ heterodimer; the resultant GroEL-αβ and SR1-αβ complexes were purified by FPLC gel filtration. Identical amounts of the GroEL-αβ or the SR1-αβ complex were incubated with GroES and trypsin in the presence or absence of nucleotides for 10 min at 23 °C. Digestion mixtures were analyzed by SDS-PAGE (upper panel) and Western blotting (lower panel) (Fig. 2). In the absence of GroES and nucleotide, the αβ heterodimer bound to GroEL was digested completely after incubation of the complex with trypsin (Fig. 2, both panels, lane 2). The heterodimer was also digested when GroES alone (lane 3), or GroES and Mg-ATP (lane 5) were added to the GroEL-αβ complex (Fig. 2, both panels). Under these conditions, GroES was unable to form a stable complex with GroEL, resulting in the degradation of the αβ heterodimer by the protease. The smaller bands under GroEL, which are stained with Coomassie Blue (upper panel), do not represent the undigested αβ heterodimer, as indicated by the absence of cross-reacting materials in Western blotting with the combined anti-α and anti-β antibodies as probes (Fig. 2, lower panel, lanes 3 and 5). These smaller bands, which migrate slower than GroES, are proteolytic products of GroEL. In the presence of Mg-ADP and GroES (lane 4), a significant portion of the heterodimer bound to GroEL was protected from trypsic digestion (Fig. 2, both panels). The amounts of α and β subunits resistant to trypsin digestion were 56.3 and 39.7%, respectively, of the starting material (lane 1) as determined by densitometry of the Western blot (Fig. 2, lower panel). The data are explained by the fact that GroES binds equally to either the cis or the trans ring of GroEL, resulting in approximately half of the cis cavity being capped by GroES. Only the αβ heterodimer inside the GroES-enclosed cis cavity is resistant to trypsic digestion.

Under identical conditions, the SR1-αβ complex was digested with trypsin in the presence of GroES with or without nucleotides. Only in the presence of GroES and Mg-ATP, where a stable SR1-GroES complex was found (3), was the SR1-bound αβ heterodimer resistant to trypsic digestion (Fig. 2, both panels, lane 10). The α and β subunits protected from the protease digestion (lane 10) were 89.4 and 101.4% of the starting materials (lane 6), respectively, as measured by densitometry of the Western blot (Fig. 2, lower panel). The results corroborate that 86-kDa αβ native-like heterodimer, despite its large size, fits inside the single SR1 cavity encapsulated by GroES, similar to that observed with GroEL.

Unfolded 86-kDa α-11-β Fusion in GroEL and SR1 Cavities Cannot Be Capped by GroES—Similar protease protection assays were carried out with the complex formed between urea-unfolded α-11-β fusion and GroEL or SR1. Incubation of the urea-denatured α-11-β fusion with GroEL or SR1 produced stable GroEL-α-11-β and SR1-α-11-β complexes, which were isolated as single species by FPLC gel filtration (data not shown). As shown in Fig. 3, the α-11-β fusion bound to GroEL was digested completely as indicated by Coomassie Blue staining, even when both GroES and Mg-ADP were present (lane 4). These conditions facilitate the formation of a stable GroEL-GroES-ADP complex. The data indicate that unfolded α-11-β fusion, despite its similar size to the αβ heterodimer, cannot be enclosed by GroES inside GroEL cavities. As positive controls, the unfolded α-11-β fusion bound to GroEL was also digested completely when GroES (lane 2) or the nucleotide (lane 3) was absent or when GroES and Mg-ATP were present (lane 5) (Fig. 3). In the same experiment, the unfolded α-11-β bound to the single-ring SR1 was also digested completely by trypsin in the presence of GroES and Mg-ATP (Fig. 3, lane 10). In the presence of Mg-ATP, GroES binds to unoccupied SR1 to form a stable complex. The data indicate that unfolded α-11-β in the SR1 cavity prevents the capping of SR1 by GroES, resulting in degradation of the unfolded α-11-β fusion. The bands under GroEL (lanes 3–5) and SR1 (lanes 8–10) are degradation products of the respective chaperonin (Fig. 3), similar to those shown in Fig. 2. These bands only appear when GroES is present, which is consistent with the finding that interactions between GroEL and GroES result in conformational changes in the former (20).

Both Subunits of the αβ Heterodimer Remain inside the SR1 Cavity Enclosed by GroES—We have shown previously that in the presence of Mg-ATP, a portion of the αβ heterodimer in the GroEL-αβ complex undergoes dissociation, resulting in the release of the α subunit into the bulk solvent (15). The SR1-αβ preparation showed that only 55% of SR1 cavities were filled with the αβ heterodimer (see Fig. 5B). It is possible that in the presence of GroES and Mg-ATP, the αβ heterodimer in the

### Table I

| Kinetic constants for wild-type αβ2 and α-11-β fusion BCKD | K<sub>v</sub> | K<sub>m</sub> | k<sub>a</sub> |
|-------------------------------------------------------------|-----|-----|-----|
| BCKD | thiamin pyrophosphate | 0.66 | 62 | 8.25 |
| α-11-β fusion | α-ketoisovalerate | 0.5 | 62 | 4.0 |

**Fig. 1.** Fluorescence emission spectra of ANS-bound wild-type BCKD (αβ<sub>2</sub>), its heterodimeric intermediate (αβ), and urea-denatured α-11-β fusion. ANS (50 μM) were mixed with wild-type His<sub>6</sub>-tagged BCKD (αβ<sub>2</sub>) in Buffer C, KSCN-induced heterodimer intermediate (αβ) in 200 mM KSCN, or urea-denatured α-11-β fusion in 8 M urea (see “Experimental Procedures”). Following a 5-min incubation at 23 °C, the emission spectrum was recorded at the excitation wavelength of 365 nm. Each spectrum was an average of three consecutive scans and was corrected for contributions from the buffer solution.
SR1-αβ complex also dissociates into individual subunits with the released α subunit binding to a new unoccupied SR1 cavity prior to the capping by GroES. In this scenario, the individual SR1-α and SR1-β complexes would present no size constraints for GroES capping and would be protected from tryptic digestion. To rule out this possibility, remaining empty cavities in the SR1-αβ/H9251/H9252 preparation were filled by incubation with excess calcium-depleted reduced 14-kDa α-lactalbumin that binds to chaperonins with high affinity (21). Fully occupied SR1 cavities were then capped by GroES in the presence of Mg-ATP. After tryptic digestion, the levels of protected α and β subunits were determined by SDS-PAGE and Western blotting and quantified by densitometry. Fig. 4 shows that most, if not all, of the α and β subunits in the fully occupied SR1-αβ/GroES preparation are recovered at the levels of 81 and 83%, respectively, of the amount in the undigested complex. Because all SR1 cavities are occupied, if α and β subunits were released into the bulk solution, they would not be able to bind to new SR1 cavities and would therefore be degraded by the protease. The above results exclude the possibility that the heterodimer dissociates into individual α and β subunits with the α subunit escaping from the original SR1 cavity prior to capping of the same cavity by GroES.

The enclosure of the SR1-αβ complex by GroES was deciphered further. As a control, in the absence of Mg-ATP, SR1-β and excess GroES do not form a complex and migrate separately in HPLC-gel filtration (Fig. 5A, top panel). In the presence of Mg-ATP, a fraction of GroES co-migrates with SR1-β to produce a stable SR1-β-GroES complex (Fig. 5A, middle panel). Molar stoichiometry of SR1:GroES in the ternary complex is 1:0.86:0.90 as determined by scanning densitometry (Fig. 5B). The data confirm that Mg-ATP promotes the stable binding of GroES to SR1, without causing the release of the protein substrate. In a parallel experiment, the SR1-αβ/H9251/H9252 complex was incubated with excess GroES and Mg-ATP, followed by separation by HPLC-gel filtration. As also shown in Fig. 5A (bottom panel), a stoichiometric amount of GroES binds to SR1-αβ complex. The molar ratio of SR1:αβ/GroES was estimated to be 1:0.55:1.08 (Fig. 5B). The result indicates that 45% of the SR1 cavity is not occupied by the protein substrate. This was corroborated by the additional binding of calcium-depleted reduced α-lactalbumin to the same SR1-αβ preparation at a subunit ratio of SR1:α-lactalbumin = 1:0.40 (data not shown). The combined data indicate that the αβ heterodimer remains inside the same SR1 cavity during the capping of SR1 by GroES.
and that all of the SR1 cavities including those occupied by the αβ heterodimer are encapsulated by GroES.

**GroEL/GroES-dependent Refolding of Wild-type and α11-β Fusion BCKD**—Because the single-ring SR1 was unable to refold BCKD proteins, a complete system comprising the double-ring GroEL complex, GroES, and Mg-ATP was utilized in refolding studies. Incubation of the GroEL-αβ complex with GroES and Mg-ATP resulted in a recovery of 72% of BCKD activity (Fig. 6A). The activity of BCKD heterotetramer based on the amount of heterodimer present in the GroEL-αβ complex was set at 100%. The heterodimeric intermediate does not possess enzyme activity. The renaturation of BCKD activity at 30% with the GroEL-α11-β fusion complex in the presence of GroES and Mg-ATP was markedly less than that obtained with the GroEL-αβ complex. The activity of the active α11-β homodimer equivalent to the amount of the unfolded monomer in the GroEL-α11-β fusion complex was set at 100%. Essentially no BCKD activity was recovered with either the GroEL-αβ complex or the GroEL-α11-β fusion complex when Mg-ATP or GroES alone was added to the refolding mixture.

Chaperonin-mediated recovery of BCKD activity was also studied using 8 M urea-denatured wild-type BCKD or α11-β fusion protein, instead of the GroEL-protein complex, as substrate (Fig. 6B). The recovery of BCKD activity with urea-denatured wild-type BCKD was also high at 90% in the presence of GroEL/GroES and Mg-ATP. By comparison, the recovery of BCKD activity at 25% with the urea-denatured α11-β fusion protein in the presence of GroEL/GroES and Mg-ATP was significantly lower than that with urea-denatured wild-type BCKD. No BCKD activity was recovered from the denatured α11-β fusion protein when GroES was omitted from the refolding mixture.

**DISCUSSION**

The αβ heterodimeric intermediate can be isolated from *E. coli* during expression and assembly of the native BCKD heterotetramer (18). The heterodimer represents an ensemble of trapped energy minima, which in the absence of chaperonins does not dimerize to form the native heterotetramer in vitro (14). We have described previously that the heterodimer does not dissociate into monomers in a measurable equilibrium and binds to GroEL as an intact species with an apparent dissociation constant ($K_d$) of $1.1 \times 10^{-7}$ M (15). The 86-kDa heterodimeric intermediate bound to GroEL was shown to be partially protected from protease digestion in the presence of Mg-ADP and GroES (15). The data strongly suggest that GroES is capable of capping this large assembly intermediate inside the GroEL cis cavity. In the present study, we extended the investigation by determining the ability of GroES to encapsulate the heterodimeric intermediate inside the GroEL single-ring variant SR1, taking advantage of the absence of the trans ring. Mutations of R452E, E461A, S463A, and V464A in the GroEL equatorial domain prevent the back to back stacking of the two GroEL rings, resulting in the formation of the single-
The 86-kDa \( \alpha\beta \) heterodimer inside the SR1 cavity (accession number 1AON) with GroEL in green and GroES in yellow. The \( \alpha\beta \) heterodimer representation, with the \( \alpha \) subunit in red and the \( \beta \) subunit in blue, was derived from PDB coordinates (accession number 1DTW). The graphics were created using Swiss-PDB Viewer and POV-ray.

The crystal structure of GroEL-GroES-ADP, complex shows a 2-fold enlargement of the \( cis \) cavity over the \( trans \) to a volume of 175,000 \( \text{Å}^3 \) (20). This volume theoretically is capable of accommodating a globular protein of \( \sim 142 \) kDa beneath GroES, assuming a perfect fit to the actual folded protein volume (20). Nonetheless, the upper limit for an unfolded protein to be encapsulated inside the GroEL cavity by GroES has been shown to be 57 kDa in both \( in vitro \) (6) and \( in vivo \) (4, 5) studies. This size constraint of 57 kDa for an unfolded protein may reflect that an unfolded polypeptide is more extended than the fully folded protein of similar size. As a result, the space larger than the actual protein volume is needed for an unfolded protein inside the \( cis \) cavity to be encapsulated by GroES. As a case in point, we show that the urea-denatured 86-kDa \( \alpha\beta \) fusion cannot be capped by GroES inside GroEL and SR1 cavity, which is consistent with the above size limit for unfolded proteins. Capping of the native-like 86-kDa \( \alpha\beta \) monomeric intermediate has not been studied, because this species cannot be isolated. On the other hand, the \( \alpha\beta \) heterodimeric assembly intermediate with a size similar to the

![Fig. 6. Chaperonin-mediated recovery of BCKD activity with GroEL-protein intermediate complexes (A) or denatured proteins (B). A, the GroEL-\( \alpha\beta \) or the GroEL-\( \alpha\)-11-\( \beta \) complex at 0.8 \( \mu \text{M} \) was incubated with 2 \( \mu \text{M} \) GroES and 10 mM Mg-ATP at 23 °C for 16 h. BCKD activity was assayed as described previously (14). The activity of BCKD or the \( \alpha\)-11-\( \beta \) fusion equivalent to the amount of the \( \alpha\beta \) heterodimer or the unfolded \( \alpha\)-11-\( \beta \) fusion in the GroEL-protein complex was set at 100%. B, the refolding of the urea-denatured BCKD and \( \alpha\)-11-\( \beta \) fusion was carried out as described previously (13). Wild-type BCKD or the \( \alpha\)-11-\( \beta \) fusion protein without denaturation was set at 100%.

![Fig. 7. Simulated encapsulation of the 86-kDa \( \alpha\beta \) heterodimer inside the \( cis \) GroEL cavity by GroES. The ribbon representation of the GroEL-GroES-ADP, complex was derived from PDB coordinates (accession 1AON) with GroEL in green and GroES in yellow. The 86-kDa \( \alpha\beta \) heterodimer representation, with the \( \alpha \) subunit in red and the \( \beta \) subunit in blue, was derived from PDB coordinates (accession number 1DTW). The graphics were created using Swiss-PDB Viewer and POV-ray.](image-url)
α-11-β fusion possesses a native-like conformation as indicated by ANS fluorescence analysis. The three-dimensional structure of the heterodimeric intermediate has not been determined. Based on the recently solved crystal structure of the native human BCKD heterotetramer, the heterodimeric intermediate contains folded α/β domains in both α and β subunits (22). In each subunit, the central β sheet is buried by outer α helices in various orientations. Proteins with this kind of folded conformation have been shown to be dependent on chaperonin for proper folding (5). The estimated volume of 132,800 Å³ for the heterodimeric intermediate enables it to fit completely inside the GroEL cis cavity, so as to be encapsulated by GroES as illustrated by simulated packing (Fig. 7).

Volume changes upon protein unfolding may also explain the striking difference between the SR1-αβ and the SR1-α-11-β complexes with respect to their ability to be capped by GroES. The partial specific volume change $\Delta V^p$ accompanying protein denaturation can be expressed as a sum of three terms, $\Delta V^p = \Delta V_{\text{prot}} + \Delta V_{\text{sol}} + \Delta V_{\text{r}}$, where $\Delta V_{\text{prot}}$ represents changes due to the loss of intramolecular voids; $\Delta V_{\text{sol}}$ represents changes in thermal volume, which result from thermally induced mutual molecular vibrations between the unfolded protein and the solvent; and $\Delta V_{\text{r}}$ is the interaction volume that represents changes in the solvent volume resulting from interactions of water molecules with charged and polar groups of the unfolded protein (23). The terms $\Delta V_{\text{prot}}$ and $\Delta V_{\text{r}}$ are inherently negative upon protein unfolding, whereas $\Delta V_{\text{sol}}$ is a positive term associated with the increase in the accessible surface area of the unfolded protein. For a protein of 70 kDa in size, the complete unfolding results in a 30% increase in the partial specific volume mainly because of the positive change in the thermal volume, $\Delta V_{\text{sol}}$ (23). It is predicted that the larger the protein, the greater increase in the partial specific volume of the unfolded state. Chemical denaturant-induced unfolding such as urea denaturation may still result in measurable residual structures (24). However, it is reasonable to expect a significant increase in the specific volume of the urea-denatured 86-kDa α-11-β fusion protein compared with its folded counterpart, i.e. the native-like αβ heterodimer of similar size and essentially identical sequences. The enlarged volume, along with the extended structure, may account for the inability of the unfolded α-11-β fusion to be enclosed by GroES inside the SR1 or GroEL cavity.

We have shown previously that chaperonins GroEL/GroES and Mg-ATP promote dissociation/reassociation cycles of the trapped heterodimeric intermediate to facilitate its conversion to the functional heterotetramer (14, 15). The encapsulation of the heterodimeric intermediate suggests strongly that the unfolding and dissociation of the heterodimer into individual α and β subunits occurs inside the encapsulated GroEL cis cavity. The subsequent binding of Mg-ATP to the open trans ring triggers the collapse of the cis assembly. The individual α and β subunits released into the bulk solvent reassemble to produce new heterodimeric intermediates with a fraction capable of dimersizing into the native heterotetramer. The dissociation/reassociation cycle perpetuates until all the trapped heterodimeric intermediates are converted to heterotetrameric BCKD. The apparent cis folding throughout the BCKD assembly pathway explains the high levels of BCKD activity recovered with either urea-denatured BCKD or the GroEL-αβ complex as substrate. By contrast, the failure of GroES to encapsulate the unfolded α-11-β fusion dictates the chaperonin-mediated trans folding of this protein, similar to that described for 86-kDa maltose-binding protein fusion (8) and 82-kDa aconitase (9). The trans folding mechanism may confer, in part, the markedly lower recovery of BCKD activity with the GroEL-α-11-β complex than that using the GroEL-αβ complex as the starting material.

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REFERENCES

1. Sigler, P. B., Xu, Z., Rye, H. S., Burston, S. G., Fenton, W. A., and Horwich, A. L. (1998) Annu. Rev. Biochem. 67, 581–608
2. Hartl, F. U. (1996) Nature 381, 571–579
3. Weissman, J. S., Hol, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saihi, H. R., Fenton, W. A., and Horwich, A. L. (1995) Cell 83, 577–587
4. Ewalt, R. L., Hendrick, J. P., Houry, W. A., and Hartl, F. U. (1997) Cell 90, 491–500
5. Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F., and Hartl, F. U. (1999) Nature 402, 147–154
6. Sakakawa, C., Taguchi, H., Makino, Y., and Yoshida, M. (1999) J. Biol. Chem. 274, 21251–21256
7. Gordon, C. L., Sather, S. K., Casjens, S., and King, J. (1994) J. Biol. Chem. 269, 27941–27951
8. Huang, Y. S., and Chuang, D. T. (1999) J. Biol. Chem. 274, 10405–10412
9. Chaudhuri, T. K., Farr, G. W., Fenton, W. A., Rospert, S., and Horwich, A. L. (2001) Cell 107, 235–246
10. Gratlert, H., Rutkat, K., and Buchner, J. (1998) J. Biol. Chem. 273, 33035–33039
11. Bhattacharyya, A. M., and Horowitz, P. M. (2002) Biochemistry 41, 422–429
12. Wynn, R. M., Davie, J. R., Cox, R. P., and Chuang, D. T. (1999) J. Biol. Chem. 267, 12400–12403
13. Chuang, J. L., Wynn, R. M., Song, J. L., and Chuang, D. T. (1999) J. Biol. Chem. 274, 10395–10404
14. Wynn, R. M., Song, J. L., and Chuang, D. T. (2000) J. Biol. Chem. 275, 2786–2789
15. Song, J. L., Wynn, R. M., and Chuang, D. T. (2000) J. Biol. Chem. 275, 22305–22312
16. Horwich, A. L., Burston, S. G., Rye, H. S., Weissman, J. S., and Fenton, W. A. (1998) Methods Enzymol. 290, 141–146
17. Clark, A. C., Ramanathan, R., and Frieden, C. (1998) Methods Enzymol. 290, 100–114
18. Wynn, R. M., Davie, J. R., Chuang, J. L., Cote, C. D., and Chuang, D. T. (1998) J. Biol. Chem. 273, 13110–13118
19. Burns, D. M., Horn, V., Palah, J., and Yanovsky, C. (1990) J. Biol. Chem. 265, 2060–2069
20. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) Nature 388, 741–750
21. Murai, N., Taguchi, H., and Yoshida, M. (1995) J. Biol. Chem. 270, 19957–19963
22. Arvander, A., Chuang, J., Wynn, M., Truley, S., Chuang, D. T., and Hol, W. G. J. (2000) Structure 8, 277–291
23. Chalkian, T. V., and Breslauer, K. J. (1996) Biopolymers 39, 619–626
24. Dill, K. A., and Shortle, D. (1991) Annu. Rev. Biochem. 60, 795–825
Encapsulation of an 86-kDa Assembly Intermediate inside the Cavities of GroEL and Its Single-ring Variant SR1 by GroES
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