Interactions of GroEL/GroES with a Heterodimeric Intermediate during $\alpha_2\beta_2$ Assembly of Mitochondrial Branched-chain $\alpha$-Ketoacid Dehydrogenase

cis CAPPING OF THE NATIVE-LIKE 86-kDa INTERMEDIATE BY GroES*

Received for publication, March 10, 2000
Published, JBC Papers in Press, April 7, 2000, DOI 10.1074/jbc.M002033200

Jiu-Li Song‡§, R. Max Wynn§¶, and David T. Chuang‡¶

From the Departments of ‡Biochemistry and §Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235

We showed previously that the interaction of an $\alpha\beta$ heterodimeric intermediate with GroEL/GroES is essential for efficient $\alpha\beta\beta$, assembly of human mitochondrial branched-chain $\alpha$-ketoacid dehydrogenase. In the present study, we further characterized the mode of interaction between the chaperonins and the native-like $\alpha\beta$ heterodimer. The $\alpha\beta$ heterodimer, as an intact entity, was found to bind to GroEL at a 1:1 stoichiometry with a $K_D$ of 1.1 × $10^{-7}$ M. The 1:1 molar ratio of the GroEL-$\alpha\beta$ complex was confirmed by the ability of the complex to bind to a stoichiometric amount of denatured lysozyme in the trans cavity. Surprisingly, in the presence of Mg-ADP, GroES was able to cap the GroEL-$\alpha\beta$ complex in cis, despite the size of 86 kDa of the heterodimer (with a His$_6$ tag and a linker). Incubation of the GroEL-$\alpha\beta$ complex with Mg-AMP, but not AMP-PNP, resulted in the release of $\alpha$ monomers. In the presence of Mg-ATP, the $\beta$ subunit was also released but was unable to assemble with the $\alpha$ subunit, and rebound to GroEL. The apparent differential subunit release from GroEL is explained, in part, by the significantly higher binding affinity of the $\beta$ subunit ($K_D < 1.15 \times 10^{-9}$ M) than the $\alpha$ ($K_D = 1.6 \times 10^{-8}$ M) for GroEL. Incubation of the GroEL-$\alpha\beta$ complex with Mg-ATP and GroES resulted in dissociation and discharge of both the $\alpha$ and $\beta$ subunits from GroEL. The $\beta$ subunit upon binding to GroEL underwent further folding in the cis cavity sequestered by GroES. This step rendered the $\beta$ subunit competent for reassociation with the soluble $\alpha$ subunit to produce a new heterodimer. We propose that this mechanism is responsible for the iterative annealing of the kinetically trapped heterodimeric intermediate, leading to an efficient $\alpha\beta\beta$, assembly of human branched-chain $\alpha$-ketoacid dehydrogenase.

It has been established that a significant number of proteins depend on the function of pre-existing protein machinery, i.e., molecular chaperones to promote proper folding in an energy-dependent manner (1–3). Among the most well studied molecular chaperones are group I chaperonins comprising bacterial GroEL/GroES, mitochondrial Hsp60/Hsp10, and plant Cpn60/ Cpn21. GroEL is a double-ring complex with two heptameric rings of identical 57-kDa subunits stacked upon one another back-to-back (4). Each GroEL monomer contains an apical, an intermediate, and an equatorial domain. The apical domain forms a heptameric GroEL ring with a diameter of 45 Å, which allows for the binding and passage of unfolded or partially folded proteins. The equatorial domain contains the ATP-binding site and provides the interring contacts. The two domains are linked through the flexible intermediate domain. GroES is a single heptameric ring of identical 10-kDa monomers (5, 6). The structure of the GroEL$_{14}$-ADP$_7$-GroES$_7$ complex (7) confirms that binding of GroES to the apical domain of GroEL induces an en bloc domain movement observed previously in cryoelectron microscopy (8). It has been suggested that this movement results in doubling the volume of the cis ring cavity of GroEL to accommodate a polypeptide of larger than 70 kDa in size (7). In support of this suggestion, the 75-kDa methylmalonyl-CoA mutase (9), an 82-kDa fusion protein of three tandem green fluorescent proteins (10), and an 86-kDa fusion polypeptide (11) were shown to bind to GroEL, but cis capping of these proteins by GroES did not occur.

The mechanism by which GroEL/GroES promote proper folding of target proteins or synthetic peptides has been extensively studied. The productive folding occurs in the cis ring that houses the unfolded or partially folded proteins, which are sequestered from the bulk solvent by the GroES (9, 12). The folding of proteins in the GroEL cavity is driven by Mg-ATP hydrolysis in the equatorial domain of the cis ring. Both rings of the GroEL double ring complex alternate to become the cis ring during GroEL/GroES-mediated folding cycles (13). This leads to the term “two-stroke engine” for the GroEL/GroES folding machine (14, 15). Communications between the apical and equatorial domains of GroEL via the intermediate domain are essential to promote proper folding of the target protein. Mutations in the intermediate domain disrupt these communications and therefore the folding capacities of GroEL/GroES (16). The peptide binding site in the apical domain has recently been identified by NMR (17–19) and x-ray crystallography (20) using minichaperones of GroEL comprising sequences of the apical domain. These studies showed independently that the flexible groove between helices H8 and H9 is the peptide-binding site for the apical domain (21). GroEL binds to a wide range of structures ranging from the extended $\beta$-strands and $\alpha$-helices to folded states with exposed hydrophobic side chains (17, 22).

Despite the above advances, only limited information is
Interactions of GroEL/GroES with an Assembly Intermediate

FIG. 1. Assembly of the human BCKD αβ heterohexameric protein assembly. The prevailing thought is that these chaperonins assist the folding of peptides into assembly-competent monomers and that spontaneous assembly proceeds in the bulk solution (23, 24). We have revisited the question by studying folding and assembly of a natural target protein, i.e. the human mitochondrial α-ketoacid dehydrogenase (BCKD).

This thiamine pyrophosphate-dependent enzyme is a component of the mammalian mitochondrial BCKD complex (5 x 10^6 daltons in size) organized around a 24-mer dihydrolipoyl transacylase core, to which BCKD, dihydrolipoyldehydrogenase, a specific kinase, and a specific phosphatase are non-covalently attached (25). BCKD is a heterotetrameric protein comprising two 45,500-dalton α subunits and two 37,800-dalton β subunits. The crystal structure of the αβ heterotramer of human BCKD has been recently solved at 2.7 Å (26). The data provide important structural insights into the previously established assembly pathway of human BCKD (27), which proceeds through an αβ heterodimeric intermediate (Fig. 1). Interactions between α and β’ and α’ and β subunits of the two heterodimers result in the assembly of a native heterotramer. These heterologous subunit interactions are blocked by a human mutation Y393N in the α subunit. This locks the mutant enzyme in the permanent heterodimeric state (26). Our previous data showed that interactions of the αβ heterodimeric intermediate with GroEL/GroES are obligatory during αβββ assembly of BCKD (28). We subsequently showed that GroEL/GroES promote the dissociation/reassociation of the αβ heterodimeric intermediate during the reconstitution of BCKD in vitro (29). These studies demonstrate a pivotal role of the chaperonins in mediating the iterative annealing of assembly intermediates for subsequent higher order oligomerization. In the present study, we further characterize the nature of interactions between GroEL/GroES and the αβ heterodimeric intermediate to better understand the mechanism by which these chaperonins promote productive protein assembly.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Bacterial chaperone GroEL, GroES, and recombinant αβ heterotetramers of human BCKD were prepared as described previously (28). The 35S-labeled and nonlabeled αβ heterodimeric intermediates of BCKD were expressed and purified also as reported previously (29).

Binding of αβ Heterodimers to GroEL—The native BCKD heterotetramer (35 μg) was incubated in 50 μl of 50 mM potassium phosphate, pH 7.5, containing 250 mM NaCl, 400 mM KSCN, and 20 mM β-mercaptoethanol at 37 °C for 1 h. The mixture containing resultant αβ heterodimers (29) was diluted to 200 mM KSCN and extracted with Ni²⁺-NTA resin for 1 h at 23 °C. The resin was washed with 10 mM imidazole in buffer containing 2 mM phenylmethylsulfonyl fluoride and incubated in 300 μl of the same buffer or with an added 330 μg of GroEL for 4 h at 23 °C in the presence of 2 mM phenylmethylsulfonyl fluoride. After collection of the supernatant, the resin was washed, and proteins bound to the resin were eluted with 200 mM imidazole. One-twentieth volume of the supernatant and one-fifth of the eluted fractions were analyzed by SDS-PAGE.

Determination of K_D for Binding of αβ Heterodimers to GroEL—Different amounts of 35S-labeled αβ heterodimers (specific radioactivity 24,642 cpm/μg of protein) were added to GroEL (final concentration 0.94 μM) in 50 mM potassium phosphate, pH 7.5, 250 mM NaCl, 1 mM EDTA in a final volume of 50 μl. After incubation for 24 h at 23 °C, the mixture was applied to an HPLC G3000SW XL column. GroEL-containing fractions were collected, and aliquots (100 μl) were counted for radioactivity. K_D was obtained by fitting the data using the DynaFit program (30) according to the scheme: αβ + GroEL ⇄ GroEL-αβ.

Preparation of Denatured and Fully Reduced Lysozyme—Lysozyme (Sigma) at 20 mg/ml was completely denatured and reduced in 6 M guanidine hydrochloride in 100 mM potassium phosphate, pH 8.0, 150 mM dithiothreitol at 37 °C for 1 h. After adjusting the pH to 2.0 with HCl, denatured and reduced lysozyme was dialyzed against 10 mM HCl for 2–3 h and then against 0.1 M acetic acid with two changes.

Differential Release of α and β Subunits from the GroEL-αβ Complex—The GroEL-αβ complex was formed by incubating GroEL with an excess amount of His-β-tagged αβ heterodimers at 4 °C for 24 h, followed by purification on a fast protein liquid chromatography Superdex-200 column. The GroEL-αβ complex was incubated with Ni²⁺-NTA resin and 10 mM Mg-ATP in the presence or absence of GroES for 30 min at 23 °C. The resin was spun down and washed with 50 mM potassium phosphate, pH 7.5, 250 mM NaCl, and 15 mM imidazole. Proteins bound to the resin were eluted with 250 mM imidazole in the same buffer and

1 The abbreviations used are: BCKD, branched-chain α-ketoacid dehydrogenase; r-LA, reduced λ-lactalbumin (calcium-depleted); NTA, nitritotriacetic acid; AMP-PNP, 5’-adenyl-β,y-imidodiphosphate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
separated on a 10–25% sucrose density gradient spun at 210,000 × g for 16 h at 4 °C. Proteins in each fraction were analyzed by SDS-PAGE.

Limited Digestion with Proteinase K—The GroEL-αβ complex at 0.8 µg/µl was subjected to limited digestion with 4 µg/µl proteinase K at 23 °C for 10 min under different conditions, and the digestion was quenched with 4 mM phenylmethylsulfonyl fluoride. Final concentrations of the additives were as follows: Mg-ATP, 8 mM; Mg-ADP, 8 mM; and GroES, 0.4 µg/µl. Samples were analyzed by SDS-PAGE and Western blotting using combined antibodies to the α and β subunits as probes.

Subunit Release from Individual GroEL-α and GroEL-β Complexes—GroEL-His6-α and GroEL-β-His6 complexes, with a His6 tag at N and C termini, respectively, were prepared as described previously (28). The GroEL-polypeptide complex at 0.2 µg/µl was incubated with 10 mM Mg-ATP in the presence or absence of 0.1 µg/µl GroES and Ni2+-NTA at 23 °C for 1 h in a final volume of 500 µl. Proteins bound to Ni2+-NTA were eluted with 500 µl of 200 mM imidazole. Incubation mixture without Ni2+-NTA and fractions eluted from the resin were separated on a 10–30% sucrose density gradient at 210,000 × g for 16 h at 4 °C. The gradient fractions were analyzed by SDS-PAGE and the released α and β subunits in the top six fractions were quantified by densitometry. The total amount of α or β subunit in the incubation mixture at zero time served as a 100% control.

Competition of BCKD Subunits with α-Lactalbumin for Binding to GroEL—Different amounts of Ca2+-depleted α-lactalbumin (Sigma) were incubated with 0.5 mM EGTA and 2 mM dithiothreitol at 23 °C for 15 min to achieve the molten globule state (31). To the incubation mixture, the GroEL-α or the GroEL-β complex (1 mg/ml) and 0.1 mM Mg-ATP were added, and the mixture was incubated for an additional 10 min. The reformed GroEL-polypeptide complex was purified on an HPLC G4000SWXL column in 50 mM potassium phosphate, pH 7.5, 250 mM KCl, and 1 mM EDTA. The GroEL-containing fractions were collected and analyzed by SDS-PAGE and Coomassie Blue staining. The amount of the α or β subunit still bound to GroEL was quantified by densitometry. The level of the α or β subunit complexed with GroEL in the absence of α-lactalbumin was set at 100%. The amounts of the α or β subunit that remained bound to GroEL were plotted against the initial concentrations of reduced α-lactalbumin (r-LA) in the incubation mixture. Computer fitting using the DynaFit 2.14 program was based on the following models: α or β + GroEL ⇌ GroEL-α or GroEL-β (Kp1), r-LA + GroEL ⇌ GroEL-r-LA (Kp2).

Protein Concentration Determination—The protein concentrations of GroEL, GroES, and denatured lysozyme were determined spectrophotometrically using published extinction coefficient of 0.21 ml/mg/cm (32), 0.14 ml/mg/cm (33), and 2.37 ml/mg/cm (34), respectively. The other proteins were determined using the Coomassie Plus protein reagent from Pierce with absorbance read at 595 nm.

RESULTS

Stoichiometric Amount of GroEL Is Needed for Maximal Recovery of BCKD Activity—We have shown previously that interaction of the αβ heterodimer with chaperonins GroEL/GroES is an obligatory step during the αβ24 assembly of BCKD (28, 29). To further delineate the nature of this interaction, we determined the amount of GroEL required for the efficient reconstitution of BCKD with the αβ heterodimeric intermediate as a substrate. Fig. 2 shows that the maximal yield of BCKD activity was obtained when the molar ratio of GroEL to the αβ heterodimer reached approximately 1:1. The αβ Heterodimer Binds to GroEL as an Intact Entity in Solution—It was uncertain whether the αβ heterodimer bound to GroEL as an entity or as dissociated α and β monomers resulting from an equilibrium that existed between the αβ heterodimer and the dissociated monomers. We addressed this question by incubating the Ni2+-NTA-immobilized His6-αβ heterodimer (with the His6 tag on the C subunit) with GroEL (Fig. 3A). Following the incubation at 23 °C for 4 h, proteins in the supernatant and the fractions eluted from Ni2+-NTA were analyzed by SDS-PAGE. The presence of the GroEL-β complex in the supernatant would be diagnostic of the dissociation of His6-αβ heterodimer. Fig. 3B shows that in the presence of the buffer alone, the His6-α and untagged β subunits remain assembled and immobilized on Ni2+-NTA. Incubation of Ni2+-NTA-His6-αβ with GroEL did not produce the soluble GroEL-β complex to be released into the supernatant. The small amount of GroEL in the eluted fraction indicates a grossly substoichiometric level of GroEL bound to the His6-αβ heterodimer immobilized on Ni2+-NTA. The data clearly establish that the αβ heterodimer is a stable species that exists in a negligible dissociation equilibrium with monomers, so as to allow the heterodimer to bind to GroEL as an intact entity.

Stoichiometry of the GroEL-αβ Complex and Affinity of αβ Heterodimers for GroEL—The affinity of the αβ heterodimer for GroEL was measured by incubating increasing concentrations of 35S-labeled heterodimers with GroEL at 23 °C for 24 h to ensure the complete binding (29). Radioactivity in the GroEL fractions collected from HPLC was quantified to determine the concentration of the bound αβ heterodimer. The stoichiometry of GroEL to the αβ heterodimer in the GroEL-αβ complex was estimated by computer simulation. The DynaFit 2.14 program was used based on the scheme αβ + GroEL ⇌ GroEL-αβ, with one binding site for the αβ heterodimer per GroEL 14-mer. The result showed a good fit with the probability of randomness p > 5% (35). The dissociation constant (Kp) according to this scheme was 1.1 × 10−7 M (Fig. 4). A second scheme assumed that GroEL possessed two binding sites for αβ heterodimers and that binding was sequential. A computer simulation based on this second scheme did not yield meaningful constants for the second binding site in the sequence (data not shown). It was therefore concluded that each GroEL molecule can bind only one αβ heterodimer.

The GroEL-αβ 1:1 Complex Can Bind Denatured Lysozyme—The 1:1 stoichiometry of the GroEL-αβ complex established above strongly suggested that the unoccupied cavity in trans on GroEL was capable of binding small unfolded proteins. To confirm this, we incubated GroEL with a saturating amount of αβ heterodimers at 4 °C for 48 h. To the GroEL-αβ complex purified by gel filtration, denatured and fully reduced lysozyme was added, and the mixture was incubated for 2 h at 23 °C. Fig. 5A shows that lysozyme co-purifies with the GroEL-αβ complex on HPLC at a 1:1 molar ratio of lysozyme to GroEL, as analyzed by SDS-PAGE. The result indicates that the GroEL-αβ complex can still bind a stoichiometric amount of lysozyme and confirms the model in which a GroEL molecule accommodates only one αβ heterodimer.
reconstitution of BCKD

lier studies have established that GroES is essential for the fractions (E). One-twentieth volume of the supernatant (S) in the fractions eluted with imidazole. With dissociation, the GroEL-b heterodimers (29). The mixture was diluted to 200 mM KSCN and extracted m and counted for radioactivity. A fraction (750 g) were mixed with 0.94 

Different amounts of ing concentrations of the target protein.

GroEL in 50 m M potassium phosphate, pH 7.5, 250 m M NaCl, 1 m M EDTA. After a 24-h incubation at 23 °C, the mixture was applied to an GroEL-containing fractions were collected HPLC G3000SWXL column. GroEL-containing fractions were collected

35S-labeled HPLC G3000SWXL column. GroEL-containing fractions were collected in the supernatant, with His6-

A heterodimer is not protected from proteolysis in the absence of protective effect of GroES during proteinase K digestion (Fig. 5). The GroEL-aβ complex was purified by a fast protein liquid chromatography Superdex-200 column. A, denatured and reduced lysozyme in excess was added to the GroEL-aβ complex, followed by a 2-h incubation at 23 °C. The lysozyme-GroEL-aβ complex was purified by HPLC on a G3000SWXL column. Lys, lysozyme. B, the GroES-GroEL-aβ complex was formed by incubating GroES with GroEL-aβ complex in the presence of 1 mM Mg-ADP and purified by HPLC in a buffer containing 0.1 mM Mg-ADP. The subunit stoichiometry was established by densitometry.

Fig. 5. The GroEL-aβ complex can bind one molecule of lysozyme or GroES. GroEL was incubated with an excess amount of the aβ heterodimer at 4 °C for 48 h to achieve maximal binding. The GroEL-aβ complex was purified by a fast protein liquid chromatography Superdex-200 column. A, denatured and reduced lysozyme in excess was added to the GroEL-aβ complex, followed by a 2-h incubation at 23 °C. The lysozyme-GroEL-aβ complex was purified by HPLC on a G3000SWXL column. Lys, lysozyme. B, the GroES-GroEL-aβ complex was formed by incubating GroES with GroEL-aβ complex in the presence of 1 mM Mg-ADP and purified by HPLC in a buffer containing 0.1 mM Mg-ADP. The subunit stoichiometry was established by densitometry.

Cis Capping of the GroEL-aβ Complex by GroES—Our earlier studies have established that GroES is essential for the reconstitution of BCKD heterotetramers with the GroEL-aβ complex as a substrate (28). However, the size of the aβ heterodimer is 86 kDa with a His6 tag and a linker. This raises the possibility that cis capping of the GroEL-aβ complex by GroES may not occur. We investigated this question by carrying out limited digestion of the GroES-GroEL-aβ complex with proteinase K. Fig. 5B shows that in the presence of Mg-ADP, a stable GroES-GroEL-aβ ternary complex can be isolated by HPLC. The subunit stoichiometry is GroEL:aβ:GroES = 14:1:1:7, as determined by densitometry. The result indicates 1 mol of bound GroES in each mol of the GroES-GroEL-aβ complex. Fig. 6 (upper panel) shows that in the presence of Mg-ADP, where a stable GroES-GroEL-aβ complex is formed, a significant amount of the a or β subunit is protected from proteinase K digestion (lane 4) compared with the starting undigested GroEL-aβ complex (lane 1), as analyzed by SDS-PAGE and Coomassie Blue staining. Western blotting confirms the presence of 42% of the intact a or β subunits after proteinase K digestion (lane 4), against the undigested GroEL-aβ complex (lane 1) (Fig. 6, lower panel). We interpret the data to indicate that, despite the oversize of the aβ heterodimer, GroES binds to the GroEL-aβ complex equally in cis and in trans, similar to results obtained with GroEL-small polypeptide complexes (10, 11). As also shown in Fig. 6, the aβ heterodimer is not protected from proteolysis in the absence of a stable GroES-GroEL-aβ complex, when GroES and ADP are omitted (lane 2), no nucleotides are added (lane 3), or Mg-ATP instead of Mg-ADP is present (lane 5). The data show the protective effect of GroES during proteinase K digestion through cis capping of the GroEL-aβ complex.

Differential Release of a and β Subunits from the GroEL-aβ Complex—To further understand the mechanism by which chaperonins promote dissociation of the aβ heterodimer (29), we investigated the effect of nucleotides and GroES on release of the a and the β subunits from the GroEL-aβ complex. The GroEL-aβ complex with a His6 tag on the a subunit was incubated with Ni2+-NTA at zero time under different conditions. After incubation at 23 °C for 30 min, proteins eluted with imidazole from the resin were separated on a sucrose density gradient. As shown in Fig. 7 (upper panel), the Ni2+-NTA-extracted GroEL-aβ complex with no addition remained essentially intact, with a slight leaching of the a subunit from the
The GroEL-αβ complex can be capped by GroES in cis. The GroEL-αβ at 0.8 μg/ml were digested with 4 μg/ml proteinase K for 10 min at 23 °C under specified conditions, and the digestion was quenched with 4 mM phenylmethylsulfonyl fluoride. The final concentrations of the additives were as follows: Mg-ATP, 8 mM; Mg-ADP, 8 mM; GroES, 0.4 μg/ml. Samples were analyzed by SDS-PAGE with Coomassie Blue staining (upper panel) and Western blotting (lower panel) using antibodies to the α and β subunits as probes.

complex during the ultracentrifugation. In the presence of Mg-ATP, a significant amount of the His6-tagged α subunit was released from the GroEL-αβ complex and sedimented as Ni2+-NTA-extractable monomers near the top of the gradient (middle panel, fractions 3–5). However, no β subunit was detected across the sucrose density gradient. Similar experiments were performed in the presence of Mg-ADP or AMP-PNP, but no release of α monomers was observed (data not shown). The results indicate that ATP hydrolysis, and not binding of the nucleotide, is necessary and sufficient for the dissociation of the αβ heterodimer. Significantly, in the presence of both Mg-ATP and GroES, a fraction of the β subunit is released from GroEL and folds into an assembly-competent conformation capable of association with the α subunit. The reformed αβ heterodimer with a His6-tagged α subunit can now be extracted with Ni2+-NTA (Fig. 7, lower panel).

Higher Level Release of the α Subunit than the β from Individual GroEL-α and GroEL-β Complexes—To compare the extent of the α and β subunits released from individual GroEL-polypeptide complexes, these complexes were incubated with Mg-ATP, with or without GroES. The subunit released from GroEL was separated on a sucrose density gradient and expressed as a percentage of that present in the starting GroEL-polypeptide complex. As shown in Fig. 8, in the presence of Mg-ATP, approximately 13% of the α subunit were released from the GroEL-α complex after a 1-h incubation; no β subunit was discharged from the GroEL-β complex (lane 1). The addition of both Mg-ATP and GroES resulted in a sharp increase in the release of the α subunit from the GroEL-α complex (lane 2). The discharge of the β subunit from the GroEL-α complex is also increased from 0% to approximately 11% (lane 2). When Ni2+-NTA was included in the incubation mixture at zero time to trap the released subunit, the levels of the released α and β subunits from individual GroEL-polypeptide complexes with Mg-ATP alone are both significant at 89 and 55%, respectively (lane 3). Similarly, significant discharges of the α and β subunits at 84 and 63%, respectively, were observed, when both Mg-ATP and GroES were included along with the Ni2+-NTA trap (lane 4).

Higher Binding Affinity of the β Subunit than the α for GroEL—The free α subunit, but not β, was released from the GroEL-αβ complex in the presence of Mg-ATP (Fig. 7, middle panel). In the absence of Ni2+-NTA, the level of the released α subunit was higher than the β subunit with individual GroEL-α and GroEL-β complexes (Fig. 8, panels 1 and 2). These results, taken together, strongly suggested that the two BCKD subunits have different affinity for GroEL. These properties were studied using reduced and calcium-depleted α-lactalbumin (r-LA) to compete with the BCKD subunits for binding to GroEL. r-LA in the molten globule state binds to GroEL with relatively high affinity (36). Competitive binding studies were carried out by the addition of different concentrations of r-LA to the GroEL-α or the GroEL-β complex in the presence of Mg-ATP. The level of the α or β subunit remaining bound to GroEL reflects the affinity of either subunit for GroEL relative to r-LA. Fig. 9 shows that the presence of a 0–50 μM concentration of r-LA does not significantly reduce the amount of the β subunit bound to GroEL. Even at 100 μM r-LA, the level of the bound β subunit is decreased by only 10%. In contrast, the level of the bound α subunit drops precipitously over the 0–50 μM concentration range of r-LA. The data show the markedly higher affinity of the β subunit than the α for GroEL. Computer simulation gave rise to a $K_D$ value of $1.6 \times 10^{-8}$ M for the GroEL-α complex and $3.0 \times 10^{-7}$ M for the GroEL-r-LA complex. The latter constant is in agreement with the binding constant of acid-denatured α-lactalbumin with GroEL (37).

With respect to $K_D$ for the GroEL-β complex, only 10% of the β subunit was released from GroEL in the presence of a 200-fold excess of r-LA. Therefore, we estimated the $K_D$ for the GroEL-β complex to be less than $4.15 \times 10^{-9}$ M by dividing the $K_D$ for the GroEL-r-LA complex by 200.

**DISCUSSION**

The present study was designed to provide further mechanistic insights into the mode of interactions between GroEL/ GroES and the αβ heterodimeric intermediate during the α3β2 assembly of human mitochondrial BCKD. The requirement for a stoichiometric amount of GroEL to the αβ heterodimer for the maximal recovery of BCKD activity indicates that the formation of a stable GroEL-αβ complex is essential during the BCKD assembly. This is in variance with the refolding of malate dehydrogenase, where a catalytic amount of GroEL forms a transient complex with the monomeric intermediate during the refolding of homodimeric enzyme (38). The direct binding of the αβ heterodimer to GroEL as an intact undissociated entity was unequivocally established by using the Ni2+-NTA immobilized heterodimer with a His6 tag on the α subunit as a ligand (Fig. 3). If an equilibrium had existed between the immobilized αβ heterodimer and dissociated α and β subunits, the addition of exogenous GroEL would have shifted the equilibrium toward the formation of individual GroEL-α and GroEL-β complexes. The failure to recover the untagged GroEL-β complex in the supernatant ruled out the possibility that the heterodimer dissociates into individual subunits prior to binding to GroEL.

The 1:1 stoichiometry of GroEL to the heterodimer is indicated kinetically by the titration of reconstituted BCKD activity (Fig. 2) and by the direct measurement of the amount of the heterodimer bound to GroEL (Fig. 4). This 1:1 stoichiometry in the GroEL-αβ complex was further supported by the ability of the complex, which was obtained with saturating concentrations of the heterodimer, to bind one molecule of fully unfolded lysozyme. The data are consistent with a topology in which one end of the GroEL double ring complex binds the heterodimer, and the opposite end accommodates the denatured lysozyme. A similar result was also observed in GroEL complexed with a homodimeric intermediate of d-glyceraldehyde-3-phosphate dehydrogenase (39). However, the basis for the “half of the sites”
binding (39) of the native-like substrate by GroEL is presently unknown. It is possible that occupation of the cis cavity by a large folded intermediate causes a narrowing of the opening in the trans ring. The constricted orifice may allow for the passage of small unfolded polypeptides but not a second molecule of the highly structured assembly intermediate. The putative conformational alterations in the apical domain of the trans ring of the GroEL-ab complex remain to be studied by cryoelectron microscopy.

The binding of one molecule of GroES in the presence of Mg-ADP by the GroEL-ab complex, on the other hand, raises a question as to whether GroES is able to cap in cis the GroEL cavity occupied by the 86-kDa heterodimer. To date, no unfolded polypeptides larger than 70 kDa have been shown to be sequestered in cis by GroES (9–11). Therefore, the cis capping of the GroEL-ab complex by GroES, as demonstrated by the proteinase K digestion, was an unexpected finding. According to the structure of the GroEL4-ADP7-GroES7 complex, the volume of the cis cavity is estimated to be 175,000 Å³ (7, 14). These authors have suggested that the cis GroEL cavity is capable of accommodating a globular protein or even an expanded volume molten globule intermediate with a molecular mass of greater than 70 kDa. This speculation is supported by the present study, which shows that the 86-kDa heterodimer can be capped by GroES in cis. We argue that the highly structured assembly intermediate is more compact than the unfolded polypeptides. This allows for the residence of the heterodimer in the cis cavity of GroEL despite the high molecular mass of the target protein. The cis capping of the GroEL-ab complex by GroES thus facilitates efficient folding or unfolding of the heterodimer to proceed in the sequestered cavity of GroEL.

The ab heterodimer is a misfolded intermediate in a low energy state. The binding energy, provided upon the binding of GroEL to the target protein, is not sufficient to drive the folding of the heterodimer. However, the presence of GroES in the cis cavity of GroEL provides a nucleation site for the assembly of the heterodimer. This allows for the formation of a stable complex that can be released from the GroEL-ribosome complex by the addition of ATP. The GroEL-ribosome complex is then free to bind to another target protein, allowing for the propagation of the folding process.

Fig. 7. Differential release of α and β subunits from the GroEL-αβ complex. The GroEL-αβ complex (1 mg/ml) was incubated with the Ni²⁺-NTA resin with shaking at 23 °C with no addition (upper panel), 10 mM Mg-ATP (middle panel), or 10 mM Mg-ATP plus 0.2 mg/ml GroES (lower panel). After 30 min, the resin was spun down and washed. Proteins eluted with 200 mM imidazole were applied onto a 10–25% sucrose density gradient, followed by centrifugation at 40,000 rpm for 16 h at 4 °C. The gradient was fractionated, and collected samples were analyzed by SDS-PAGE.

Fig. 8. Subunit release from individual GroEL-α and GroEL-β complexes. The GroEL-α complex with an N-terminal His₆ tag on the α subunit and the GroEL-β complex with a C-terminal His₆ tag on the β subunit were prepared as described previously (28). The GroEL-polypeptide complex at 0.2 μg/μl was incubated at 23 °C for 1 h with different combinations of 10 mM Mg-ATP, 0.1 μg/μl GroES and Ni²⁺-NTA as indicated. Proteins bound to Ni²⁺-NTA were eluted with 200 mM imidazole. The mixture without Ni²⁺-NTA and fractions eluted from Ni²⁺-NTA were separated on a 10–30% sucrose density gradient, and fractions were analyzed by SDS-PAGE. The α and β subunits released from GroEL were quantitated by densitometry. Total amount of the α or β subunit in the GroEL-polypeptide complex incubated with no addition served as a 100% control.

Fig. 9. Competition of the α and β subunits with r-LA for binding to GroEL. α-Lactalbumin at the indicated concentration was incubated for 15 min at 23 °C in 50 mM potassium phosphate, pH 7.5, 0.5 mM EGTA, 2 mM dithiothreitol, 250 mM NaCl, and 2 mM MgCl₂. To the completely reduced and calcium-depleted α-lactalbumin (r-LA), the GroEL-α or GroEL-β complex (0.45 μM) and 0.1 mM Mg-ATP were added, and the mixture was incubated for an additional 10 min. The GroEL-polypeptide complex was purified on an HPLC G4000SWxl column. The amounts of the α or β subunits co-purified with GroEL were determined by SDS-PAGE and densitometry. The amount of the α or β subunits complexed with GroEL in the absence of r-LA was set as 100%.
the heterodimer and nucleotide to GroEL, probably weakens the interaction between the $\alpha$ and $\beta$ subunit but is still not enough to dissociate the heterodimer, since no released $\alpha$ subunit was observed in the presence of AMP-PNP or ADP (data not shown). In the presence of Mg-ATP alone, the $\beta$ subunit is also dissociated from GroEL but is unable to reassemble with the soluble released $\alpha$ monomer and rebinds to GroEL (Fig. 7).

Since the $\beta$ subunit is without a His$_8$ tag, the GroEL-$\beta$ complex is not extracted by Ni$^{2+}$-NTA. The preferential release of the $\alpha$ subunit by ATP hydrolysis ensures the cis capping of the resultant GroEL-$\beta$ complex by GroES, since the 37.8-kDa $\beta$ subunit is well within the size limit for this mechanism. The efficient cis folding produces the $\beta$ subunit competent for reassembly with the $\alpha$ subunit, with the regeneration of $\alpha\beta$ heterodimers separated on the sucrose density gradient. The results confirm and extend the thesis that GroEL/GroES promote the dissociation and reassociation of the $\alpha\beta$ heterodimer to facilitate the assembly of $\alpha\beta$ heterotetramer at the quaternary level (29). This mechanism is consistent with GroEL-assisted folding through the unfolding of misfolded intermediates (40).

The release of both $\alpha$ and $\beta$ subunits from individual GroEL-$\alpha$ and GroEL-$\beta$ complexes with Mg-ATP alone is indicated by the extraction of both subunits with Ni$^{2+}$-NTA (Fig. 8). However, with Mg-ATP alone, the majority of the released $\alpha$ subunit rebinds to GroEL. When both Mg-ATP and GroES are present, essentially all of the released $\alpha$ subunit stays in solution. The results indicate that GroES is required to augment the proper folding of the denatured $\alpha$ subunit. In contrast, with Mg-ATP alone, all of the $\beta$ subunit discharged from the GroEL-$\beta$ complex rebinds to GroEL. With both Mg-ATP and GroES present, only a small fraction of $\beta$ subunit is folded and exists as a soluble species. This folded $\beta$ subunit is conceivably capable of assembly with the soluble $\alpha$ subunit.

The differential dependence of the $\alpha$ and $\beta$ subunits on GroEL/GroES for folding, can be explained, at least in part, by the recently determined BCKD structure (Fig. 1). During assembly, the two homologous heterodimeric intermediates ($\alpha\beta$ and $\alpha'\beta'$) assemble into a native BCKD $\alpha\beta\beta\alpha$ heterotetramer. The $\beta$ subunit as shown in the heterodimer is divided into two distinct domains, with each domain comprising four parallel central $\beta$ strands and several $\alpha$-helices that pack against the strands in variable orientations. This conformation fits the type of structures that has recently been suggested to depend on GroEL/GroES for folding (41). The $\beta$ subunit contains mostly strands that fold slowly and need to be precisely packed into the $\alpha\beta$ architecture and thus are prone to misfold. In contrast, the $\alpha$ subunit in the heterodimer and native BCKD comprises essentially $\alpha$ helices that are less prone to misfolding. The data are consistent with the higher affinity of the $\beta$ subunit ($K_D < 4.1 \times 10^{-9} \text{ M}$) than the $\alpha$ subunit ($K_D = 1.6 \times 10^{-7} \text{ M}$). This in turn accounts for the retarded release of the $\beta$ subunit from GroEL-$\alpha\beta$ complex as compared with the $\alpha$ subunit.

Fig. 10 illustrates a working model for chaperonin-depend en $\alpha\beta$ assembly of human BCKD. Our earlier results showed that GroEL/GroES and Mg-ATP facilitate folding of the $\alpha$ and $\beta$ subunits into assembly-competent monomers on different GroEL scaffolds (28). The $\alpha\beta$ heterodimer initially formed is not capable of spontaneous dimerization and is termed a “bad” dimer. The slow binding ($t_{1/2} = 54.8 \text{ min}$) of bad dimers to GroEL produces stable GroEL-$\alpha\beta$ complexes (29). In the presence of Mg-ATP alone, the $\alpha$ subunit released from the GroEL-$\alpha\beta$ complex exists as soluble monomers. In contrast, the $\beta$ subunit discharged from the complex is not capable of reassembly with the $\alpha$ subunit and therefore rebinds to GroEL. In the presence of both Mg-ATP and GroES, the $\beta$ subunit undergoes multiple rounds of cis folding, resulting in the release of the assembly-competent $\beta$ subunit that reassociates with the $\alpha$ monomer to produce new heterodimers. A fraction of the regenerated $\alpha\beta$ heterodimers are productive or “good” dimers that are capable of rapid ($t_{1/2} = 1.83 \text{ min}$) spontaneous dimerization into native heterotetramers, followed by a slow ($t_{1/2} = 2.93 \text{ min}$) conversion to active heterotetramers (29). However, the reformulated heterodimers are partitioned between good and bad dimers with the latter having to rebind to GroEL. Thus, the cycle perpetuates until all the bad dimers are converted to good dimers. This model describes a central role of GroEL/GroES in protein assembly through iterative annealing of nonproductive assembly intermediates.
Acknowledgments—We thank George Lorimer for helpful discussions and Tsan Xiao for assistance in preparing molecular graphics of human BCKD structures.

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