GroEL/GroES Promote Dissociation/Reassociation Cycles of a Heterodimeric Intermediate during $\alpha_2\beta_2$ Protein Assembly

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While the mechanism of GroEL/GroES-mediated protein folding has been extensively studied, the role of these chaperonins in oligomeric protein assembly remains poorly understood. In the present study, we investigated the interaction of the chaperonins with an $\alpha\beta$ heterodimeric intermediate during the $\alpha_2\beta_2$ assembly of human mitochondrial branched-chain $\alpha$-ketoacid dehydrogenase/decarboxylase (BCKD). Incubation of the recombinant His$_6$-tagged BCKD in 400 mM KSCN for 45 min at 23 °C caused a complete dissociation of the $\alpha_2\beta_2$ heterotetramers into inactive $\alpha\beta$ heterodimers. Dilution of the denaturant resulted in a rapid recovery of BCKD independent of the chaperonins GroEL/GroES. Prolonged incubation of BCKD in 400 mM KSCN resulted in the generation of nonproductive or “bad” heterodimers, which were unable to undergo spontaneous reactivation but capable of binding to GroEL to form a stable GroEL-$\alpha\beta$ complex. Incubation of this complex with GroES and Mg-ATP led to the slow reactivation of BCKD with a second-order rate constant $k_2 = 480 \text{ M}^{-1} \text{s}^{-1}$. Mixing experiments with radiolabeled and unlabeled protein substrates provided direct evidence that GroEL/GroES promote dissociation and subunit exchange between bad heterodimers. This was accompanied by the transformation of bad heterodimers to their “good” or productive counterparts. The good heterodimers were capable of spontaneous dimerization to initially form an inactive heterotetrameric species, followed by conversion to active heterotetramers. However, a large fraction of bad heterodimers were regenerated and re-bound to GroEL. The cycle was perpetuated until the reconstitution of active BCKD was complete. Our data support the thesis that chaperonins GroEL/GroES mediate iterative annealing of nonproductive assembly intermediates at the quaternary structure level. This step is essential for an efficient subsequent higher order oligomerization.

Bacterial chaperonins GroEL/GroES, which are homologues of mitochondrial chaperonins Hsp60/Hsp10, respectively, have been shown to promote energy-dependent protein folding both in vitro and in vivo (1, 2). GroEL is a double-ring complex with two heptameric rings of identical 57-kDa subunits stacked upon one another back-to-back (3). Each GroEL monomer contains an apical, an intermediate, and an equatorial domain. The apical domains form a heptameric GroEL ring with a diameter of 45 Å, which facilitates the passage of unfolded or partially folded proteins. GroES is a single heptameric ring of identical 10-kDa monomers (4, 5). The structure of the GroEL$_{14}$-ADP$_7$-GroES$_7$ complex confirms that binding of GroES to the apical domain of GroEL induces an en bloc domain movement, resulting in doubling the volume of the cis-ring cavity of GroEL, which allows accommodation of a polypeptide of up to 70 kDa in size (6, 7). The mechanism of GroEL/GroES-mediated protein folding has been extensively investigated using small proteins. It has been suggested that the asymmetric GroEL/GroES complex, with only one end of GroEL capped by GroES, elicits productive protein folding (8, 9). Studies with ornithine transcarbamylase have established that the polypeptide is productively released only from the cis-ring of GroEL sequestered by GroES (9). GroEL/GroES-mediated protein folding proceeds through multiple rounds of binding and release from GroEL until the protein attains its native conformation (2, 10). It has been a subject of controversy with respect to the mechanism by which GroEL/GroES support correct protein folding. The “Anfinsen cage” model dictates that the folding of a polypeptide occurs in the protected hydrophobic environment of GroEL cavities (7, 11, 12). The iterative annealing mechanism is the one in which GroEL/GroES catalyzes the unfolding of misfolded polypeptides or conformers, and actual folding ensues in the bulk solution (13–16). However, the two models are not necessarily mutually exclusive. Upon capping by GroES, the GroEL cavity lining transforms from hydrophobic to hydrophilic as the result of a 90° torque along the long axis (7). This conformational change conceivably induces either folding or unfolding of polypeptides bound to the GroEL cavity.

Despite the fact that the concept of GroEL/GroES as molecular chaperones originated from the discovery that they are essential for bacterial phage $\lambda$ prophage assembly (17, 18), the actual role of these chaperonins in oligomeric protein assembly remains poorly understood. The current dogma is that GroEL/GroES aid in folding of nascent or unfolded polypeptides into assembly-competent monomers and that spontaneous assembly occurs in the bulk solution (19, 20). However, recent studies have shown that GroEL is capable of interacting with native-like conformers. For example, GroEL was shown to facilitate a conversion of a highly structured Fab intermediate to a functional species through a process not involving unfolding (21). Hydrogen-deuterium exchange of small proteins barnase (22) and cyclophilin A (23) showed that global unfolding occurs upon binding of these native conformers to GroEL. Recently, GroEL was reported to trap both dimeric and monomeric un-
folding intermediates of citrate synthase induced by heat denaturation (24). The interactions of GroEL with native-like conformers raise the question whether GroEL/GroES play an active role in oligomeric assembly beyond the folding of polypeptides into assembly-competent monomers.

To investigate the putative role of chaperonins in protein assembly, we have used human mitochondrial branched-chain α-ketoacid dehydrogenase/decarboxylase (BCKD) as a model system. BCKD is a thiamine pyrophosphate-dependent enzyme comprising two 45.5-kDa α subunits and two 37.8-kDa β subunits. BCKD is a component of the mitochondrial BCKD complex (molecular mass, 5 × 10^6 kDa), which organizes around a cubic core comprising 24 dihydrolipoyl transacylase subunits, to which BCKD, dihydrolipoyl dehydrogenase, a specific kinase, and a specific phosphatase are attached through ionic interactions (25). The BCKD complex is deficient in patients with heritable maple syrup urine disease, resulting in severe acidosis and mental retardation (26). Mutations in the α subunit were shown to impair BCKD assembly in maple syrup urine disease patients (27).

We have shown previously that co-transformation of GroEL/GroES into *Escherichia coli* expressing mammalian BCKD subunits resulted in an over 500-fold increase in the yield of the active BCKD heterotetramer, compared with the single transformant without overexpression of chaperonins (28, 29). Recently, we reported *in vitro* reconstitution of human BCKD with an absolute requirement for GroEL/GroES and Mg-ATP (30, 31); however, the kinetics of BCKD reconstitution was markedly slower than that of chaperonin-mediated refolding of other proteins. GroEL-αβ complex resulting from binding of a large 85-kDa αβ heterodimeric intermediate to GroEL was also isolated. Incubation of this complex with GroEL and Mg-ATP led to the reconstitution of native heterotetramers. In the present study, we further characterize the interaction of GroEL/GroES with the heterodimeric intermediate. We show that GroEL/GroES promote multiple cycles of dissociation/reassociation of the heterodimers. This slow step is indispensable in priming the highly structured intermediate for subsequent heterotetrameric assembly.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Human His₆-tagged BCKD—The recombinant His₆-tagged BCKD protein was efficiently expressed in *E. coli* strain CG-712 (ES ts) from the pHisT-hE1 plasmid by co-transformation of the pGroESL plasmid overproducing chaperonins GroEL and GroES, as described previously (28, 30). Assembled active His₆-tagged BCKD heterotetramers were isolated from cell lysates and purified on a Ni²⁺-NTA-derivatized Sepharose CL-6B column (Qiagen, Chatsworth, CA), as also described previously (27).

Estimation of Molecular Masses by Dynamic Light Scattering—Dynamic light scattering measurements were carried out using a DP-801 DLS instrument from Protein Solutions (Charlottesville, VA) interfaced with a Mitsuba computer. Samples of BCKD (0.6 mg/ml) with or without hydrogenase/decarboxylase; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

The distribution of diffusion coefficients derived from the autocorrelation function was determined using a method of nonparametric least squares laplacian transformation (regularization) contained in the Dynamics 4.0 software package from Protein Solutions. The hydrodynamic (Stokes) radius (Rg) of the molecules was derived from the Stokes-Einstein equation (Equation 1),

$$ R_g = kT/6\pi nD $$

where n, k, and T are viscosity, the Boltzmann constant, and the absolute temperature, respectively (32).

The corresponding molecular mass (M) was calculated according to Equation 2,

$$ R_g = [(3/4\pi n)vM_N a_h h^2]/13 $$

where N_a is Avogadro’s number, v (0.73 cm³/mol) is the partial specific volume of the protein, and h (1.3) is the hydration for soluble proteins.

The frictional coefficient (γ) was obtained from the Einstein-Sutherland equation (Equation 3),

$$ D = kT/\gamma $$

where D denotes the diffusion coefficient at 22 °C, with the remaining variables defined as above.

A frictional coefficient for a sphere (fₜ) was also determined for the KSCN-induced heterodimer using Equation 4, with the variables defined as above.

$$ f_t = 6\pi n(3M/[N_a bh^2])^{1/3} $$

**Spontaneous Reactivation of KSCN-denatured Human BCKD—** Prior to the denaturation of BCKD with KSCN, it was necessary to eliminate all possible reducing equivalents. BCKD heterotetramers were precipitated with 2.5 mM ammonium sulfate and resuspended in an inactivation buffer minus KSCN (50 mM potassium phosphate, pH 7.5, 2.5 mM KCl, and 1 mM EDTA). Following the KSCN treatment, BCKD was quickly diluted 10-fold into a spectrophotometric assay mixture consisting of 100 mM potassium phosphate, pH 7.5, 2.0 mM MgCl₂, 0.2 mM thiamine pyrophosphate, 0.1 mM EDTA, and 0.1 mM 2,6-dichlorophenolindophenol (DCPIP) as reported previously (33). Reactivation of BCKD was allowed to proceed in the assay mixture at 37 °C for various lengths of time. At different reactivating times, α-ketoisovalerate (0.1 mM) was added to the incubation mixture to start the enzyme reaction. BCKD activity was monitored by decrease in absorbance at 600 nm due to the reduction of DCPIP.

**Simulation of the Time Course for Spontaneous Reactivation of KSCN-denatured Human BCKD—** Data from the recovery of native BCKD activity were fitted using the enzyme kinetics program DynaFit 2.14 (34) on a Power Macintosh computer. The kinetic model used for the simulation was based on the following reaction scheme (Equation 5),

$$ k_1 \frac{2\alpha\beta}{2} \rightarrow [\alpha\beta] \rightarrow \alpha \beta $$

where the reaction begins with two inactive heterodimers (αβ) that upon dilution of KSCN first assemble into an inactive heterotetrameric species, ([αβ]₃), followed by conversion into the active heterotetramer. The second- and first-order rate constants k₁ and k₂, respectively, were determined by plotting the recovered BCKD activity as a function of time and running multiple iterations with the DynaFit 2.14 program until the simulated curve agreed with the experimental data. Application of the test for randomness of groupings in the sequence of signs of residuals (BCKD data points (C) in Fig. 3) gave a probability of randomness of 13%. Because this is greater than the limit of significance 5%, randomness may be assumed, indicative of a good fit.

**Reconstitution of BCKD with the GroEL-αβ Complex Formed between GroEL and KSCN-induced Heterodimers—** His₆-tagged BCKD (2 μM) was incubated with 400 mM KSCN for 45 min at 23 °C. The incubation mixture was diluted 2-fold into a potassium phosphate buffer, pH 7.5, containing 250 mM KCl and 1 μM GroEL. Following incubation for 1–3 h at 23 °C, the GroEL-αβ complex formed was purified by HPLC on a G3000SWxl column. The column buffer contained 50 mM potassium phosphate, pH 7.5, 250 mM KCl, and 1 mM EDTA. Fractions containing the GroEL-αβ complex were collected, pooled, and concentrated in a Millipore (Bedford, MA) Ultrafree-15 filter device with a 50 kDa cut-off Biomax membrane. Reconstitution of BCKD was carried out as described previously (28) by incubation of 1 μM purified GroEL-αβ with 2 μM GroES and 10 mM Mg-ATP. At indicated times, aliquots were collected and assayed for BCKD activity using a radiochemical method that measured the decarboxylation rate of α-keto[¹⁴C]isovalerate. This assay utilized the reconstituted BCKD complex activity in the presence of excess recombinant dihydrolipoyl transacylase and dihydrolipoyl dehydrogenase components as described previously (30).
Expression and Purification of an 35S-labeled Heterodimeric Intermediate—The pH10-T-11 and the pGroESL plasmids were co-transformed into E. coli CG-712 cells grown at 37 °C in the C2 minimal medium as described previously (27). Following induction for expression of BCKD subunits with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 5 min, 7 mM of the 35S-labeled Met/5Cys mixture (Amersham Pharmacia Biotech) was added to the 200 ml of the C2 medium, and induction of BCKD subunit expression was continued for 3 h. Lysates prepared from harvested cells were extracted with Ni2+-NTA resin and washed with a lysis buffer containing 15 mM imidazole (27). Proteins bound to the resin were eluted with the same buffer containing 250 mM imidazole. Eluted 35S-labeled His6-tagged αβ heterodimers and His6-tagged αα, ββ heterotetramers were separated by FPLC on a HiLoad Superdex 200 column (2.6 × 60 cm) (30). Typically, the specific radioactivity of purified 35S-labeled heterodimers was between 25,000–35,000 cpm/μg protein. Nonlabeled heterodimers were prepared in a similar fashion except that no radiolabel was added to the growth media (30).

Removal of the His6 Tag from 35S-labeled Heterodimers—To remove the His6 tag, 0.5–1.0 mg of 35S-labeled His6-tagged αβ heterodimers in 50 mM Tris-HCl, pH 8.0, 5.0 mM EDTA, and 1 mM dithiothreitol were incubated at 4 °C overnight with the recombinant tobacco-etch virus protease (Life Technologies, Inc.) at a 1:200 weight ratio of tobacco-etch virus protease:protein. The tobacco-etch virus protease cleaved at a highly specific sequence (LDNLFFQ I S) present between the His6 tag and the N terminus of the α subunit. The released His6 tag was removed by incubation of the digestion mixture with excess Ni2+-NTA resin. Following centrifugation, the supernatant containing untagged 35S-labeled heterodimers was collected.

Reconstitution of BCKD with Mixed His6-tagged and Untagged GroEL-Heterodimer Complexes—GroEL-His6-αβ and GroEL-αα, ββ complexes were prepared by incubation of nonlabeled His6-αβ and untagged 35S-labeled αα, ββ heterodimers and His6-tagged αα, ββ heterotetramers were separated by FPLC on a HiLoad Superdex 200 column (2.6 × 60 cm) (30). Typically, the specific radioactivity of purified 35S-labeled heterodimers was between 25,000–35,000 cpm/μg protein. Nonlabeled heterodimers were prepared in a similar fashion except that no radiolabel was added to the growth media (30).

RESULTS
Reversible Denaturation of Human BCKD with KSCN—We previously reported that the reconstitution of denatured human BCKD ααββ heterotetramers proceeds through an αβ heterodimeric intermediate (30). It was also shown that chaperonins GroEL/GroES promote the conversion of inactive heterotetramers into active heterotetramers (30). To further investigate this chaperonin requirement for conversion of the heterotetrameric intermediate, we used a mild chaotropic agent, KSCN, to denature His6-tagged human BCKD. The enzyme was incubated in 400 mM KSCN at 23 °C for different lengths of time. Fig. 1 shows a rapid inactivation of BCKD by KSCN treatment, with enzyme activity essentially lost after 30 min of incubation. The assembly state of KSCN-treated BCKD was analyzed by FPLC gel filtration and dynamic light scattering. Fig. 2A shows that after an incubation in 400 mM KSCN for 45 min, BCKD is eluted as an 85-kDa heterodimeric species (peak 2) from an FPLC Superdex-200 column. The 1:1 heterodimeric subunit composition was confirmed by SDS-PAGE (data not shown). The untreated BCKD (Fig. 2A, peak 1) shows a molecular mass of 170 kDa with an elution profile clearly separated from that of the KSCN-induced heterodimers. The average Stokes radii of BCKD heterotetramers (ααββ) and KSCN-generated heterodimers (αβ) as determined by dynamic light scattering are shown in Fig. 2B. The molecular mass of BCKD heterotetramers as derived from the Stokes radius, followed by normalization to known molecular markers, is 91 kDa. These size measurements established that after an incubation with 400 mM KSCN for 45 min, BCKD heterotetramers were completely dissociated into heterodimers. Additionally, the fractional coefficient (f) for the heterodimers was calculated using Equation 2 and compared with the fractional coefficient for a perfect sphere (f0) by using Equation 4. The fractional coefficient ratio (f/f0) (7.96 × 10−10 g cm−1 s−1/5.90 × 10−9 g cm−1 s−1) was calculated to be 1.35. This ratio suggests that the KSCN-induced heterodimers are nonspherical molecules. A hydrated sphere would have a fractional ratio of f/f0 = 1.12.

To assess whether the KSCN-generated heterodimers were capable of spontaneously converting into heterotetramers, re-folding studies were carried out by diluting the sample by 10-fold into a BCKD assay mixture containing 0.1 mM DCPIP. BCKD activity was measured spectrophotometrically by monitoring the decrease in absorbance at 600 nm due to reduction of DCPIP. Fig. 3 shows a rapid spontaneous reactivation of BCKD, upon dilution of the denaturant. The sigmoidal refolding kinetics supports the reaction scheme depicted in Equation 5. The scheme specifies that the KSCN-induced heterodimers first rapidly assemble into an inactive heterotetrameric species with a second-order rate constant ki = 4.55 × 108 M−1 s−1 (t1/2 = 1.83 min for the initial concentration of 2 μM for the heterodimer) (see legend to Fig. 3). This is followed by a spontaneous conversion to native heterotetramers, which is a slow process.
The second-order rate constant ($k_2$) is 1 order of magnitude faster than that ($290 \text{ M}^{-1} \text{s}^{-1}$) obtained with 8 M urea-denatured BCKD in the presence of GroEL/GroES and Mg-ATP (30). It is noteworthy that the presence of a 2-fold molar excess of GroEL had no significant effect on the reactivation of KSCN-treated BCKD (Fig. 3). The results are consistent with the thesis that heterodimers induced by KSCN under these conditions were unable to bind GroEL.

**Interaction of Nonproductive Heterodimers with GroEL/GroES**—The above results show that the mild KSCN treatment produced a class of heterodimeric intermediates that is capable of spontaneous dimerization independent of chaperonins GroEL/GroES. To dissect the effect of prolonged KSCN treatment, BCKD exposed to KSCN at different lengths of time was analyzed by tryptophan fluorescence spectroscopy. As shown in Fig. 4, there is a significant decline in tryptophan fluorescence emission as a function of time following exposure of BCKD to KSCN. Spectra 4–6 (1–4.5 h of KSCN exposure) represent tryptophan emission of KSCN-generated heterodimers. This is based on the observation that the dissociation of heterotetramers to heterodimers is complete after a 45-min incubation (Fig. 2). The steady reduction in tryptophan fluorescence emission from the 1-h to the 4.5-h treatment indicates a continuous unraveling of the heterodimeric structure. However, there are no free or soluble dissociated BCKD and β monomers because unassembled subunits would aggregate in the absence of chaperonins GroEL/GroES (30).

BCKD heterodimers produced by exposure to KSCN for different time periods were analyzed for their abilities to spontaneously assemble into active heterotetramers. Fig. 5 shows an inverse relationship between the length of time for BCKD exposure to KSCN and the recovery of enzyme activity. After treatment with KSCN for 3 h, the dissociated heterodimeric intermediate lost >95% of its ability to renature into active BCKD. During the entire treatment period, BCKD remained in the heterodimeric state as analyzed by sucrose density gradient centrifugation (data not shown). The data indicate that there is a time threshold for KSCN treatment, beyond which the productive heterodimers are transformed into a species that is no longer able to undergo spontaneous reactivation. In the next series of experiments, BCKD exposed to 400 mM KSCN for 45 min was further incubated for 1–3 h in the presence of 200 mM KSCN and GroEL. The extended incubation...
tion produced a stable GroEL-αβ complex that was further purified by HPLC. Addition of GroES and Mg-ATP to the purified GroEL-αβ complex resulted in the reactivation of BCKD (Fig. 6). However, the kinetics of recovered BCKD activity reverted to a very slow one, with a rate constant of 480 M⁻¹ s⁻¹ (Fig. 6). This rate constant is similar to that obtained with a different GroEL-αβ complex, formed between GroEL and a heterodimeric intermediate isolated from E. coli lysates (30).

GroEL/GroES-mediated Dissociation and Subunit Exchange between Nonproductive Assembly Intermediates—To address whether GroEL/GroES cause the disassembly of heterodimers, a refolding experiment was performed using two different heterodimeric intermediates, i.e. nonlabeled His₅-αβ and ³⁵S-labeled untagged αβ⁺ heterodimers as substrates. The former was prepared by growing E. coli overexpressing His₅-α and untagged β subunits in the C2 medium, and the His₅-αβ heterodimers were isolated from the bacterial lysate as described previously (30). ³⁵S-Labeled His₅-αβ⁺ heterodimers were made similarly except that cells were grown in the C2 medium containing ³⁵S-labeled Met/Cys. The His₅ tag was subsequently removed by digestion of the purified ³⁵S-labeled His₅-αβ⁺ heterodimer with the tobacco-etched virus protease, which cleaved at the specific site LDNLQYFG Q 

* intermediate species, which is extractable with the resin, is diagnostic for a subunit exchange as a result of dissociation and reassociation of the heterodimers (Fig. 7A). One cannot discern the subunit exchange from no exchange by examining the heterotetramers, as they will contain radiolabel in both subunits in either scenario after dimerization of the heterodimers (not shown). Fig. 7B shows phosphorimaging following SDS-PAGE of sucrose density gradient fractions of aliquots collected from the refolding mixture at different times. At the 2-h time point, trace amounts of the ³⁵S-labeled β⁺ subunit were detected in the heterodimeric intermediate (fractions 3–6). The ³⁵S-labeled β⁺ subunit in the heterodimers became most prominent at the 12-h time point. The signal in the β⁺ subunit was converted largely from that in the heterodimer to the one in the heterotetramer after an 18 h incubation. The appearance of radiolabeled α and β subunits in heterotetramer correlated with recovery of activity in BCKD (data not shown).

The result from the substrate mixing experiment unequivocally showed that chaperonins GroEL/GroES mediate dissociation and subunit exchange of nonproductive heterodimers.

Slow Binding of Heterodimers to GroEL—To measure the on-rate for binding of heterodimers to GroEL, ³⁵S-labeled
indicated times, aliquots were removed and extracted with Ni2+
change between nonproductive BCKD heterodimers.
A, flow chart. Nonlabeled His6-
resin. His6-tagged protein species bound to the resin were eluted with
extracted refolding mixtures. GroEL-His6-
erodimers, is the 35S-labeled
of subunit exchange, resulting from dissociation/reassociation of het-
intermediates formed were extracted with Ni2+
BCKD at 23 °C in the presence of GroES and Mg-ATP. Heterodimeric
complexes at a 1:1 stoichiometry were used as substrates to reconstitute
with GroEL were purified by HPLC. The combined GroEL-heterodimer
complexes were incubated with the resin. B, phosphorimaging of Ni2+-NTA
extracted refolding mixtures. GroEL-His6-
and GroEL-
β* after incubation
with GroES and Mg-ATP resulted in the formation of a new
complex on a sucrose density gradient (Fig. 9A). The assembly state of the isolated heterodimers (peak fractions 4 and 5) remained unchanged during further purification on the sucrose density gradient (Fig. 9B). Incubation of the purified heterodimers with GroEL resulted in the formation of a new
αβ complex, which was stable and sedimented near the
bottom of the gradient (Fig. 9C). Addition of GroES and Mg-
ATP to the regenerated GroEL-αβ complex, followed by a 16-h
incubation, led to the release of new heterodimers from GroEL,
with the formation of a predominant heterotetrameric species
(peak fractions 6–8, Fig. 9D). The appearance of heterotetramers
is correlated with the recovery of BCKD activity (data not
shown). These results indicate that repetitive interactions of
the heterodimeric intermediate with GroEL/GroES are neces-
sary for its conversion to the active heterotetramer.

**DISCUSSION**

The present study was undertaken to provide further mech-
ansic insight into the interaction between chaperonins
GroEL/GroES and the heterodimeric intermediate during αβ2
assembly of human BCKD (30). We postulated that the het-
rodimeric species isolated from E. coli or the refolding reaction
mixture, which were able to bind to GroEL, were trapped
misconformers. These misconformers presumably possess a
high activation energy barrier (∆G‡) that prevents them from
escaping from their trapped states. The question arose of
whether a productive heterodimeric intermediate exists with a
conformation favorable for spontaneous dimerization. To test
this hypothesis, we used a relatively mild chaotropic reagent,
KSCN, to partially unfold BCKD without disrupting the het-
rodimeric assembly. Here, we show that KSCN partially dis-
sociates BCKD into productive heterodimers that are capable
of rapid spontaneous dimerization to initially produce inactive
heterotetramers, which are subsequently converted to active

**FIG. 7.** GroEL/GroES-mediated dissociation and subunit ex-
change between nonproductive BCKD heterodimers. A, reaction
flow chart. Nonlabeled His6-αβ and [35S]Met/Cys-labeled untagged αβ
heterodimers were prepared as described under “Experimental Pro-
cedures.” The resultant GroEL-His6-αβ and GroEL-αβ* heterodimers
were incubated with GroEL were purified by HPLC. The combined
GroEL-heterodimer complexes at a 1:1 stoichiometry were used as substrates to reconstitute
BCKD at 23 °C in the presence of GroES and Mg-ATP. Heterodimeric
intermediates formed were extracted with Ni2+-NTA. Only in the event of subunit exchange, resulting from dissociation/reassociation of heter-
odimers, is the [35S]labeled β subunit in the released His6-αβ het-
erodimers extracted with the resin. B, phosphorimaging of Ni2+-NTA
extracted refolding mixtures. GroEL-His6-αβ and GroEL-αβ* at 0.5 μM
each were incubated with 2 μM GroES, and 10 mM Mg-ATP at 23 °C.
At indicated times, aliquots were removed and extracted with Ni2+-NTA
resin. His6-tagged protein species bound to the resin were eluted with
250 mM imidazole and separated on 10–25% sucrose density gradients
for 18 h at 4 °C. Collected fractions (0.7 ml) were analyzed by SDS-PAGE, followed by phosphorimaging. The molecular mass markers used for calibration were as follows: ovalbu-
min (44 kDa), bovine serum albumin (66 kDa), dihydrolipoyl dehydro-
genase (110 kDa), aldolase (158 kDa), catalase (232 kDa), and GroEL (540 kDa).

His6-αβ heterodimers were incubated with GroEL at 23 °C.
Aliquots collected at different times during the incubation were
separated by HPLC. Radioactivity contained in the GroEL
fractions was determined and converted into moles of bound αβ
dimers. Unexpectedly, the binding of the heterodimers to
GroEL exhibited exceedingly slow kinetics with an on-rate of
k = 621 M−1 s−1 (Fig. 8). A mutant heterodimer carrying a
Y393N maple syrup urine disease substitution in the α subunit
was also found to bind to GroEL at a similarly slow rate (data
not shown). These rate constants are markedly slower than
that for the binding of unfolded α monomers to GroEL (data not
shown).

**Repeated Interactions of Heterodimers with GroEL—**A ques-
tion arose as to whether one round of GroEL/GroES-facilitated
dissociation and subunit exchange of nonproductive het-
rodimers was sufficient to convert all heterodimeric interme-
diates to native heterotetramers. To investigate this problem, heterodimers released from the GroEL-αβ complex after a 2-h
incubation with GroES and Mg-ATP were separated from the
GroEL-αβ complex on a sucrose density gradient (Fig. 9A). The assembly state of the isolated heterodimers (peak fractions 4 and 5) remained unchanged during further purification on the sucrose density gradient (Fig. 9B). Incubation of the purified heterodimers with GroEL resulted in the formation of a new
αβ complex, which was stable and sedimented near the
bottom of the gradient (Fig. 9C). Addition of GroES and Mg-
ATP to the regenerated GroEL-αβ complex, followed by a 16-h
incubation, led to the release of new heterodimers from GroEL,
with the formation of a predominant heterotetrameric species
(peak fractions 6–8, Fig. 9D). The appearance of heterotetramers
is correlated with the recovery of BCKD activity (data not
shown). These results indicate that repetitive interactions of
the heterodimeric intermediate with GroEL/GroES are neces-
sary for its conversion to the active heterotetramer.

**FIG. 8.** Kinetics of slow binding of nonproductive BCKD heter-
odimers to GroEL. [35S]Met/Cys-labeled His6-tagged heterodimers
(0.36 mg/ml, 24,501 cpm/μg protein) were prepared and purified as
described under “Experimental Procedures.” The [35S]-labeled het-
erodimer (0.488 μM) and GroEL (1.2 μM) were incubated at 23 °C in 50
mM potassium phosphate, pH 7.5, 250 mM NaCl, 1 mM EDTA. At
different times, 50-μl aliquots were removed and applied to an HPLC
G3000SWxl column. GroEL-containing fractions were collected and
counted for radioactivity. The second-order on-rate (k on) was obtained
by computer fitting of the experimental data using the DynaFit 2.14
program.

The present study was undertaken to provide further mech-
ansic insight into the interaction between chaperonins
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this hypothesis, we used a relatively mild chaotropic reagent,
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odimeric assembly. Here, we show that KSCN partially dis-
sociates BCKD into productive heterodimers that are capable
of rapid spontaneous dimerization to initially produce inactive
heterotetramers, which are subsequently converted to active
was separated on a 10–25% sucrose density gradient by centrifugation. The GroEL–E. coli 10 mM Mg-ATP. After incubation for 2 h at 23 °C, the refolding mixture was separated on a 10–25% sucrose density gradient by centrifugation (C). The GroEL–α complex is currently under investigation by cryo-electron microscopy. It is of interest that Tyr-393 and Phe-364 residues in the C-terminal region of the α subunit are in contact with Asp-328 and Tyr-313, respectively, in the β subunit of the neighboring heterodimer (42). Human mutations Y393N and F364C in the α subunit dislocate the interface between two heterodimers, resulting in permanently trapped bad heterodimers (27) capable of also binding to GroEL. However, interaction with GroEL/GroES did not alter the assembly state of the mutant heterodimer (data not shown).

Chaperonins have been shown to be capable of binding to highly native-like structures ranging from monomeric folding intermediate (36) to heat-denatured and guanidine-HCl denatured homodimers (24, 37) to native proteins (22, 38). Proton-deuterium exchange experiments indicate that the binding energy alone is sufficient to cause global unfolding of barnase (22) and cyclophilin A (38). Incubation of the complex formed between GroEL and heat-denatured citrate synthase homodimers with GroES and Mg-ATP was shown to result in the conversion of inactive to active homodimers (24). However, chaperonin-mediated unfolding/dissociation of the denatured homodimer of citrate synthase has not been demonstrated. In the present study, the BCKD assembly intermediate comprising distinct α and β subunits offers an appropriate system to study the unfolding activity of GroEL. Results of the subunit exchange between bad heterodimers (Fig. 7) provide the direct evidence for GroEL/GroES-catalyzed dissociation/reassociation of the heterodimeric intermediate. Thus, our results show that the partial unfolding, as mediated by GroEL/GroES, of heterodimers occurs at the quaternary structural level and is required for the transformation of bad heterodimers to their good counterparts. This reaction represents an integral forward step during the αβ2 assembly of BCKD. In contrast, the interaction of GroEL with the inactive Fab intermediate leads to the activation of the Fab fragment without dissociation of the disulfide bond-linked subunits (21). The dissociation of the BCKD heterodimer can be caused by GroEL alone in the presence of Mg-ATP, with the α subunit released as folded monomers and the β subunit remaining bound to GroEL.2 Although it is less than clear at present, the reassociation of α and β subunits presumably occur mainly in the bulk solution. The subsequent necessary interactions of the reformed heterodimer with GroEL (Fig. 9) conforms to the principle of multiple cycles of action by GroEL/GroES in mediating protein folding (10, 15).

A specific feature associated with the chaperonin-dependent reconstitution of BCKD heterotetramers is its unusually slow kinetics (30). Its second-order rate constant (290 s⁻¹) is 2 orders of magnitude slower than that for the refolding of malate dehydrogenase (39). The second-order rate constant (4.55 × 10³ s⁻¹) for the rapid spontaneous dimerization of KSCN-induced good heterodimers into the inactive heterotet-

Fig. 9. Multiple rounds of binding and release of BCKD heterodimers from GroEL. The GroEL–αβ complex was prepared by incubation of GroEL with the heterodimer isolated from E. coli lysates followed by purification by FPLC as described previously (30). The refolding mixture contained 1 µM GroEL–αβ complex, 2 µM GroES, and 10 mM Mg-ATP. After incubation for 2 h at 23 °C, the refolding mixture was separated on a 10–25% sucrose density gradient by centrifugation at 210,000 × g for 18 h at 4 °C. Protein species were collected in 0.7 ml fractions and analyzed by SDS-PAGE (A). The heterodimeric species (fractions 4 and 5) were pooled, concentrated, and refractionated on a 10–25% sucrose density gradient (B). The purified heterodimer (1 µM) from B was incubated with (1 µM) GroEL at 23 °C for 6 h, followed by sucrose density gradient centrifugation (C). Fractions containing the GroEL–αβ complex (fractions 12–15) were pooled. The GroEL–αβ complex (1 µM), GroES (2 µM) and Mg-ATP (10 mM) were added, followed by incubation for 12 h at 23 °C. The refolding mixture was analyzed by sucrose density gradient centrifugation and SDS-PAGE as described above (D). The molecular weight markers used are as described in Fig. 7.
the GroEL-heterodimers bind to GroEL to produce are incapable of dimerization to form active BCKD heterotetramers. These “bad” heterodimers bind to GroEL to produce the GroEL$\alpha\beta$ complex (reaction 2). In the presence of GroES and Mg-ATP, GroEL promotes the dissociation of bad heterodimers. The ensuing reassociation of the separated $\alpha$ and $\beta$ subunits results in the production of both “good” (reaction 3) and bad (reaction 4) heterodimers. The good heterodimers are short-lived and undergo rapid spontaneous dimerization to form an inactive BCKD heterotetrameric intermediate (reaction 5), as indicated by the brackets. The final step (reaction 6) represents the spontaneous conversion of the inactive heterotetramers to active BCKD. The regenerated bad heterodimers are subjected to iterative cycles of annealing until the conversion of all heterodimeric intermediates to BCKD is complete.

The GroEL-GroES-mediated dissociation/reassociation cycles (27, 30) strongly suggest that the slow binding of the nonproductive or bad assembly intermediate to GroEL is the rate-limiting step during the GroEL-GroES-mediated reconstitution of BCKD. The slow binding to GroEL precludes the calculation of moles of ATP required for the slow GroEL/GroES-assisted conversion of heterodimers to heterotetramers based upon the rate of ATP hydrolysis by GroEL (16). The slow on-rate for binding to GroEL also explains the accumulation of free bad heterodimers during chaperonin-mediated reconstitution of BCKD in vitro and expression in E. coli (27, 30).

The results presented in this study support the iterative annealing mechanism of chaperonin-mediated protein folding (16) with an extension from the tertiary to the quaternary structure level. As shown in Fig. 10, a large portion of heterodimeric intermediates, formed by the association of $\alpha$ and $\beta$ subunits released from individual GroEL (reaction 1), possess nonnative structure and do not proceed to produce native BCKD heterotetramers. These nonproductive or bad heterodimers represent an ensemble of intermediates with nonnative low energy minima by virtue of incorrect quaternary interactions. The fraction of good heterodimers capable of undergoing spontaneous dimerization is negligibly small during the early phase of BCKD reconstitution in vitro (30). The reasons for the predominance of bad heterodimers during early BCKD assembly are not entirely clear but may be related to the slower folding of the $\beta$ subunit than the $\alpha$ into assembly-competent conformations. This discordance in folding rates may result in a biased assembly of heterodimeric misconformers. GroEL rescues these kinetically trapped misconformers by forming the GroEL-$\alpha\beta$ ternary complex (reaction 2). In the presence of Mg-ATP, GroEL/GroES promote annealing of the heterodimeric intermediates through a dissociation/reassociation cycle. Unlike the dissociation of soluble malate dehydrogenase aggregates (39), stoichiometric amounts of GroEL are needed for this cycle (data not shown). This is because the dissociated $\beta$ subunit is transient and needs to rebind to GroEL unless assembled with the $\alpha$ subunit. The annealing process produces both good (reaction 3) and bad (reaction 4) heterodimers. The good heterodimers are subjected to spontaneous dimerization with the initial production of heterotetramers (reaction 5), followed by slow conversion to active heterotetramers (reaction 6). The latter step presumably involves the final conformational adjustments that are required for the inactive heterotetramer to assume a native BCKD structure. The good heterodimers are short-lived species due to the rapid kinetics of dimerization and therefore cannot be isolated. The bad heterodimers rebind to GroEL thereby perpetuating the dissociation/reassociation cycle of the assembly intermediate until the assembly of BCKD heterodimers is complete. The significantly slower rate constant of reaction 2 than of reactions 5 and 6 indicates that the step for binding of bad heterodimers to GroEL is the pace-setter for the annealing cycle. Thus, our results describe a central role of GroEL/GroES during oligomeric protein assembly by facilitating the ATP-dependent iterative annealing of kinetically trapped quaternary intermediates.

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