Mechanisms for GroEL/GroES-mediated Folding of a Large 86-kDa Fusion Polypeptide in Vitro*

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Our understanding of mechanisms for GroEL/GroES-assisted protein folding to date has been derived mostly from studies with small proteins. Little is known concerning the interaction of these chaperonins with large multidomain polypeptides during folding. In the present study, we investigated chaperonin-dependent folding of a large 86-kDa fusion polypeptide, in which the mature maltose-binding protein (MBP) sequence was linked to the N terminus of the α subunit of the decarboxylase (E1) component of the human mitochondrial branched-chain α-ketoacid dehydrogenase complex. The fusion polypeptide, MBP-α, when co-expressed with the β subunit of E1, produced a chimeric protein MBP-E1 with an (MBP-α)2β2 structure, similar to the α2β2 structure in native E1. Reactivation of MBP-E1 denatured in 8 M urea was absolutely dependent on GroEL/GroES and Mg2+-ATP, and exhibited strikingly slow kinetics with a rate constant of 376 M–1 s–1, analogous to denatured untagged E1. Chaperonin-mediated refolding of the MBP-α fusion polypeptide showed that the folding of the MBP moiety was about 7-fold faster than that of the α moiety on the same chain with rate constants of 1.9 × 103 s–1 and 2.95 × 104 s–1, respectively. This explained the occurrence of an MBP-αGroEL binary complex that was isolated with amylose resin from the refolding mixture and transformed Escherichia coli lysates. The data support the thesis that distinct functional sequences in a large polypeptide exhibit different folding characteristics on the same GroEL scaffold. Moreover, we show that when the α-GroEL complex (molar ratio 1:1) was incubated with GroES, the latter was capable of capping either the very ring that harbored the 48-kDa (His)6-α polypeptide (in cis) or the opposite unoccupied cavity (in trans). In contrast, the MBP-α-GroEL (1:1) complex was capped by GroES exclusively in the trans configuration. These findings suggest that the productive folding of a large multidomain polypeptide can only occur in the GroEL cavity that is not sequestered by GroES.

Molecular chaperones are a class of proteins which assist folding of other proteins in the cell by preventing or reversing aggregation caused by off-pathway folding reactions (1–3). Many of the chaperones are heat shock proteins (Hsp), named for their induced synthesis in cells during heat-shock stress. Group I chaperonins (4, 5) include GroEL/GroES in bacteria, Hsp60/Hsp10 in eukaryotic mitochondria, and ribulose-5P carboxylase-binding protein/cpn21 in plant chloroplasts (6, 7). These proteins exhibit remarkable structural and functional conservation from bacteria to plants to humans (8–10). The conserved function in the chaperonin family is the basis that bacterial GroEL and GroES are widely used in promoting refolding of various proteins, including those from mitochondria and chloroplasts both in vitro and in Escherichia coli (1–3). The biogenesis of eukaryotic mitochondrial matrix proteins is proposed to follow a complex chaperone-mediated pathway (11–13). Unfolded subunit polypeptides are imported into mitochondria as aided by Hsp70 family chaperones, and the final stage of folding and assembly of mitochondrial oligomeric proteins is promoted by chaperonins Hsp60/Hsp10 (11–13).

Our laboratory has previously shown the obligatory role of GroEL and GroES in facilitating folding and assembly of the decarboxylase (E1) and the transacylase (E2) components of human branched-chain α-ketoacid dehydrogenase complex in vitro (14, 15) and in E. coli (16). E1 is a thiamine pyrophosphate-dependent enzyme, consisting of two 45-kDa α and two 38-kDa β subunits (17). In an earlier study (18), we overexpressed MBP-E1 in which the mature sequence of maltose-binding protein (MBP) was fused to the N terminus of the mature α subunit (abbreviated MBP-α). Although MBP was shown to rapidly and efficiently refold in vitro without chaperonins (18), the overexpression of MBP-E1 in E. coli required co-expression of GroEL/GroES (16, 19). The results have established that fusion of a spontaneously refolded protein to the α sequence from E1 does not change chaperonin-dependent folding characteristics of the latter sequence. The native conformation of a large polypeptide is often folded into several compact regions or domains (20). Previous studies with spontaneously refolded proteins, e.g. tryptophan synthetase (21, 22), dihydrofolate reductase (23), and aspartokinase-homoserine-dehydrogenase (24, 25), show that individual domains in the entire polypeptide chain refold differentially and can be expressed as soluble functional units, suggesting that the domain alone is a folding unit. Co-translational independent domain folding in eukaryotes has been recently demonstrated using a Ras-dihydrofolate reductase fusion polypeptide

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1 The abbreviations used are: Hsp, heat shock proteins; CDTA, trans-1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid; E1, branched-chain α-ketoacid decarboxylase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoyl dehydrogenase; MBP, maltose-binding protein; MBP-α, the fusion polypeptide between MBP and the α subunit of E1; Ni-NTA, Ni-nitrilotriacetic acid; PK, proteinase K, PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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(26). Molecular chaperones do not contain information for specifying correct folding. The information for folding into the functional three-dimensional structure of a protein is solely present in its amino acid sequence (27). Thus, one can reasonably expect that independent domain folding also occurs on the GroEL scaffold.

The crystal structure of unliganded GroEL double-ring complex has indicated that, in the absence of GroES, a polypeptide of up to ~35 kDa in size can be accommodated within a single ring of GroEL (28, 29). Binding of GroES, however, induces a large conformational change in GroEL, leading to an approximate doubling of the volume in the central cavity of that ring. This allows the accommodation of polypeptides of ~70 kDa in size (30). Because of the size constraint of the GroEL cavity, it has been proposed that GroES promotes the productive release of polypeptides larger than 70 kDa from a trans configuration, in which GroES and the unfolded polypeptide bind to the opposite rings of GroEL (31). However, evidence for the productive trans release of large polypeptides from GroEL is lacking.

In the present study, we investigate GroEL/GroES-assisted refolding of the large 86-kDa MBP-α fusion polypeptide in vitro. The kinetic data show that the MBP moiety folds appreciably more rapidly than the α subunit. This explains the isolation of an MBP-α/GroEL binary complex from the MBP-E1 refolding mixture and E. coli lysates with amylose resin. Moreover, we provide evidence that GroES can only cap the MBP-α/GroEL complex in trans in relation to the fusion polypeptide. This is in contrast to that observed in the α-GroEL complex where GroES binds to GroEL in both cis and trans configurations. These findings provide a paradigm for independent domain folding during chaperonin-assisted folding of large multidomain polypeptides.

**EXPERIMENTAL PROCEDURES**

**Materials—**CG712 (an E. coli Es™ strain) and the expression plasmid pGroESL overexpressing GroEL and GroES (32) were generous gifts from Drs. George Lorimer and Anthony Gatenby of DuPont Experimental Station (Wilmington, DE). The pH1 plasmid carrying pHisT-hE1 plasmids were grown overnight at 37 °C in YTGK media containing 125 μg/ml ampicillin and 50 μg/ml chloramphenicol. The overnight culture was diluted 6:100 into 1 liter of YTGK medium with added antibiotics. The overnight culture was diluted 6:1000 into 1 liter of YT medium containing 10 mM KCl, 1% glycerol, and antibiotics.

**Preparation of MBP-α Fusion Polypeptides—** Unless otherwise stated, at indicated times, aliquots (1 ml) of the refolding mixture was withdrawn and the refolding reaction was terminated by the addition of 50 mM CDTA to chelate Mg2+ ions. The refolding mixture was incubated with 0.5 ml of amylose resin for 10 min. After a brief spinning, the supernatant was removed and the resin was washed three times with 1 ml of the refolding buffer. Proteins bound to the resin were eluted twice with 0.5 ml of the refolding buffer containing 20 mM maltose. The eluted protein sample was loaded on a 10–30% sucrose density gradient, and separated by centrifugation at 210,000 × g for 18 h.

**Determination of Rate Constants for the E1 Refolding Reaction (36, 37)—** To determine rate constants for the folding of MBP- and α moieties, the data were fit to the first-order rate equation: ln[A] = ln[A]₀ - k₅t, where [A] and [A]₀ represent the molar concentration of the α moiety at a given time; [A]₀ = initial concentration of the reactant; k₅ = rate constant for the folding reaction; and t = the reaction time. To determine rate constants for the reconstitution of E1 activity from denatured E1 or MBP-E1, the data were fit to second-order rate equation, kt = 1/[A] - 1/[A]₀, where [A] = α, MBP-α or -β monomers at a given time. The percent of E1 activity recovered was used to calculate the amount of E1 or MBP-E1 tetratetramers formed. The same amount of E1 or MBP-E1 not treated with the denaturant was incubated in the refolding buffer for 24 h, and the enzyme activity served as a 100% refolding control. The percent of folded E1 or MBP-E1 tetratetramers at different time points was calculated. The concentration of remaining E1 monomers was derived by subtracting a 2-fold concentration of the E1 tetratetramer from the initial concentration of the α (or β) monomers. The assumption was that the α and β subunits participating in the E1 refolding reaction either stayed as monomers or complexed with each other to form heterodimers or heterotetramers according to the assembly pathway: 2 α + 2 β → 2 αβ → αβ₂ (14). The concentrations of α and β monomers were identical at any time during the refolding. Therefore, V = k[V₀]₀/[β] is treated as V = k[α][β] = k₅[α][β] = k₅αβ₂ = k₅(αβ)₂, where k₅ = 2 (14). The second-order equation, kt = 1/[A] - 1/[A]₀, was derived after integration.

**Protease K Digestion of Folding Intermediates—** At indicated times of the refolding reaction, a 100-μl aliquot of the refolding mixture containing 0.5 μM MBP was removed. The sample was digested with 4 μg/ml PK for 10 min at 22 °C, and the digestion then quenched with 5 mM FMSF. The same amounts of native E1 and MBP-E1 were also treated with PK and served as a 100% refolding control. The protease-digested samples were precipitated with 7.5% trichloroacetic acid containing 125 μg/ml sodium deoxycholate, and analyzed by SDS-PAGE and Western blotting.

**Preparation of MBP-α GroEL Complexes—** (His)₅₀-GroEL and β GroEL Complexes—MBP-E1 (3 mg) was denatured in 400 μl of the denaturation buffer (50 mM sodium acetate, pH 4.5, and 8 mM urea) for 15 min. The denatured protein was incubated with 200 μl of the carboxymethyl-Sepharose CL-6B resin (Pharmacia) equilibrated with the denaturation buffer for 15 min. The resin was washed with the denaturation buffer containing 60 mM KCl. Denatured MBP-α bound to the resin was eluted with the denaturation buffer containing 300 mM KCl. The eluted MBP-α was repeatedly diluted in 50 mM potassium PO₄, pH 7.5, containing 100 mM KCl and 8 mM urea and concentrated in a Microcon-30 concentrator (Amicon). The denatured protein was diluted into 50 mM potassium PO₄, pH 7.5, containing 100 mM KCl and 1:1 molar ratio of GroEL to make 1:1 stoichiometric substrate-GroEL complexes. The mixture was separated on a 10–25% sucrose density gradient and the MBP-α GroEL complex collected. The preparation of (His)₅₀-GroEL and β GroEL Complexes was described (14).

**RESULTS**

**Kinetic Measurements for Renaturation of E1 and MBP-E1—** The refolding of untagged E1 or MBP-E1 denatured in 8 mM urea was initiated by dilution of the denatured protein into a refolding buffer at 22 °C containing GroEL/GroES and Mg²⁺-ATP.
Fig. 1. Kinetics for GroEL/GroES-dependent reconstitution of untagged human E1. Recombinant human E1 was denatured in 8 M urea and reconstituted at 22 °C in the presence of GroEL/GroES and Mg\(^{2+}\)-ATP as described under “Experimental Procedures.” The final concentrations of denatured E1 tetramers for refolding, following a 100-fold dilution, were 0.25 µM (□), 0.125 µM (○), and 0.0625 µM (○). The data at 0.125 and 0.0625 µM of E1 were fit to the second-order function to yield rate constants. Folding efficiencies at 0.25, 0.125, and 0.0625 µM denatured E1 were 68, 60, and 83%, respectively. Δ GroEL, GroES, or Mg\(^{2+}\)-ATP omitted. One milliunit (mU) was defined as 1 nmol of \(^{14}\)CO\(_2\) evolved per min.

Fig. 2. Time course for chaperonin-mediated reactivation of urea-denatured MBP-E1. The denaturation and reconstitution at 22 °C of the MBP-E1 fusion protein were as described in the legend to Fig. 1. The concentrations of the denatured MBP-E1 tetramer for refolding were 0.2 µM (□), 0.1 µM (○), and 0.05 µM (○). The data at all three protein concentrations were fit to the second-order function to produce folding rate constants. Folding efficiencies at 0.2, 0.1, and 0.05 µM denatured MBP-E1 were 67, 99, and 84%, respectively. Δ GroEL, GroES, or Mg\(^{2+}\)-ATP absent.

Fig. 3. Resistance of refolded MBP to proteinase K digestion. MBP denatured in 8 M urea was diluted into the refolding buffer (final concentration 0.5 µM) and refolded for 1 h at 22 °C in the absence (panel A) or presence (panel B) of GroEL/GroES/Mg\(^{2+}\)-ATP. The refolding mixture was subsequently treated with 4 µg/ml PK also at 22 °C for 10 min and the digestion quenched with PMSF. The digested MBP refolding mixture (100 µl in panel A and 240 µl in panel B) was separated on a 10–25% sucrose density gradient and fractions analyzed on 12% SDS gels, followed by Coomassie Blue staining.

MBP. As shown in Fig. 4, refolding of unconjugated MBP reached 99.8 and 99.9% of the native MBP control in the absence or presence of GroEL/GroES/Mg\(^{2+}\)-ATP, respectively. The plateau levels for refolding of the MBP moiety on the MBP-α fusion polypeptide from the denatured MBP-E1 were 68.3 and 27.2% in the presence or absence of GroEL/GroES/Mg\(^{2+}\)-ATP, respectively (Fig. 4). The rate constants for the first-order reaction for MBP folding in the absence or presence of chaperonins were 0.020 and 0.036 s\(^{-1}\), respectively. They were in the same order of magnitude as previously reported value of 0.025 s\(^{-1}\) for spontaneous MBP refolding (39), as determined by tryptophan fluorescence measurements. The rate constants for refolding of the MBP moiety on MBP-α in the absence or presence of chaperonins are 1.1 × 10\(^{-3}\) and 1.9 × 10\(^{-3}\) s\(^{-1}\), respectively (Fig. 4). These values are
were summed and the maximum at the 4-h time point was converted to (MBP-E1)(fractions 3 and 4) and assembled (MBP with GroEL/GroES/Mg2+ aliquots were withdrawn and digested with 4 m concentrations and separated (His)6-MBP by liquid chromatography gel filtration (14). The radioactivity associated with protein bands was quantified by PhosphorImaging. □, unfused MBP without chaperonins; ○, fused MBP with GroEL/GroES/Mg2+ -ATP; Δ, MBP moiety in MBP-a fusion without chaperonins; □, MBP moiety in MBP-a fusion with GroEL/GroES/Mg2+ -ATP.

FIG. 4. Folding kinetics of unfused and fused forms of MBP in the presence or absence of GroEL/GroES. The urea-denatured MBP or MBP-E1 was refolded at 0.25 and 0.125 μM, respectively, at 22 °C with or without GroEL/GroES/Mg2+ -ATP. At different times, aliquots were withdrawn and digested with 4 μg/ml PK at 22 °C for 10 min. The same amount of native MBP or MBP-E1 treated with PK was used as a control (100% standard). After quenching with PMSF, the digested mixture was separated on SDS-PAGE, transferred to Immobilon-P membrane (Millipore), and probed with the MBP antibody and 125I-protein A. The radioactivity associated with protein bands was quantified by PhosphorImaging. □, unfused MBP without chaperonins; ○, unfused MBP with GroEL/GroES/Mg2+ -ATP; Δ, MBP moiety in MBP-a fusion without chaperonins; □, MBP moiety in MBP-a fusion with GroEL/GroES/Mg2+ -ATP.

an order of magnitude lower than those measured with unlinked MBP.

Folded State of the α Moiety during Renaturation of MBP-E1—Since the folded α subunit does not possess enzyme activity, the refolding of the α moiety on MBP-a was monitored by the release of MBP-a monomers from GroEL and subsequent assembly of the folded α moiety with β to form the (MBP-a)β dimer or the (MBP-a)β2 tetramer (14). Aliquots collected at different times during refolding were separated on a sucrose density gradient and fractions analyzed by SDS-PAGE and Coomassie Blue staining. The reaction mixture was not extracted with amylose resin so that measurement of the released MBP-a fusion was not dependent on the refolded MBP moiety. Fractions 3 to 8 corresponding to MBP-a monomers, (MBP-a)β dimers, and (MBP-a)β2 tetramers were quantified by densitometry scanning (Fig. 5A). As shown in Fig. 5A, at the 0-min time point, all of MBP-a or -β was complexed with GroEL and sedimented close to the bottom of the gradient (fractions 13–15). The bands in fractions 3 and 4 were dissociated GroEL monomers. At the earlier time points (5 min to 1 h), the majority of MBP-a was present as unassembled MBP-a monomers (fractions 3 and 4) and assembled (MBP-a)β dimers (fractions 4–6). At later time points (2 to 24 h), the dimers were gradually converted to (MBP-a)β2 tetramers, which peaked in fractions 6–8. The identities of these E1 folding intermediates as separated by the sucrose density gradient were confirmed by fast protein liquid chromatography gel filtration (14). The densities of all folded MBP-a species (fractions 3–8) were summed and the maximum at the 4-h time point was expressed as 100%. The percent of folded MBP-a species was plotted against the folding time to depict the refolding kinetics of the α moiety. The folding of the α moiety reached a plateau in about 4 h (Fig. 5B), which was much slower than the MBP (15 min) on the same fusion polypeptide (Fig. 4). The rate constant for folding of the α moiety fit to the first-order reaction was 2.95 × 104 s–1. This rate constant is independent of assembly with the β subunit, since both unassembled and assembled MBP-a species were counted. To measure the percent formation of dimers and tetramers during the refolding, subtotals of the normalized MBP-a signal in fractions 4–5 (in the dimeric state) and fractions 7–8 (in the tetrameric state) were expressed as percents of the maximal intensity and plotted against the folding time (Fig. 5C). No significant E1 activity was recovered (about 10%) until 2 h when an appreciable amount of tetramers accumulated. The time course for recovery of E1 activity was in approximate parallel with that for the formation of MBP-E1 tetramers (Fig. 5C). The slight discordance between the two rates was the result of two separate experiments. The data show that the tetramer, but not the dimer, is the enzymatically active form, and confirm that the slow process in E1 refolding is the formation of tetrads from dimers (14).

An MBP-a-GroEL Complex Isolated from the Transformed Bacterial Lysate—During the overexpression of the MBP-a fusion polypeptide with GroEL/GroES in E. coli, a significant amount (10 mg/liter) of the MBP-a-GroEL complex (Fig. 6, lanes 11–14) and aggregated MBP-a (Fig. 6, lane 15) were isolated with the amylose resin when analyzed by sucrose density gradient centrifugation. The data were consistent with the notion that the MBP moiety was folded and capable of binding to the resin. The α moiety on the MBP-a fusion was apparently unfolded and associated with GroEL, resulting in the formation of the MBP-a-GroEL complex. The MBP-a polypeptide was a preterminated translation product. The MBP-a-GroEL complex isolated from the E. coli lysate produced enzymatically active MBP-E1 when incubated with the β-GroEL complex and GroES-Mg2+ -ATP (data not shown). The result indicated that the MBP-a-GroEL complex was a productive folding intermediate when MBP-a was expressed in E. coli.

To ask whether the MBP-a-GroEL intermediate also occurred during MBP-E1 folding, aliquots collected from the refolding mixture were extracted with amylose resin and separated on a sucrose density gradient. As shown in Fig. 7, at the 10-min time point, when the majority of the MBP moiety was folded (Fig. 4) with a less than 50% maximum of the α moiety folded or complexed with β (Fig. 5 B), a significant amount of the MBP-a-GroEL complex was isolated with the resin (fractions 9–12) (Fig. 7, upper panel). At the 16-h time point, only (MBP-a)β dimers (fractions 3 and 4) and (MBP-a)β2 tetramers (fractions 5 and 6) were extracted with the resin (Fig. 7, lower panel). The data establish that the MBP-a-GroEL complex is indeed a productive folding intermediate during the refolding of the MBP-a moiety on the MBP-a fusion. The trans-MBP-a-GroEL Complex as a Folding Competent Species—Due to the size constraint associated with the GroEL cavity, we suspected that during the folding reaction GroES can cap the MBP-a-GroEL complex only at the open end unoccupied by the large 86-kDa fusion polypeptide, resulting in the exclusive trans configuration. To address this question, we first studied the binding of a smaller polypeptide 48-kDa (His)6-protein A. The binding to the resin. The MBP-a moiety on the MBP-a fusion was unfolded and associated with GroEL, resulting in the formation of the MBP-a-GroEL complex. The MBP-a polypeptide was a preterminated translation product. The MBP-a-GroEL complex isolated from the E. coli lysate produced enzymatically active MBP-E1 when incubated with the β-GroEL complex and GroES-Mg2+ -ATP (data not shown). The result indicated that the MBP-a-GroEL complex was a productive folding intermediate when MBP-a was expressed in E. coli. The trans-MBP-a-GroEL Complex as a Folding Competent Species—Due to the size constraint associated with the GroEL cavity, we suspected that during the folding reaction GroES can cap the MBP-a-GroEL complex only at the open end unoccupied by the large 86-kDa fusion polypeptide, resulting in the exclusive trans configuration. To address this question, we first studied the binding of a smaller polypeptide 48-kDa (His)6-protein A. The binding to the resin. The MBP-a moiety on the MBP-a fusion was unfolded and associated with GroEL, resulting in the formation of the MBP-a-GroEL complex. The MBP-a polypeptide was a preterminated translation product. The MBP-a-GroEL complex isolated from the E. coli lysate produced enzymatically active MBP-E1 when incubated with the β-GroEL complex and GroES-Mg2+ -ATP (data not shown). The result indicated that the MBP-a-GroEL complex was a productive folding intermediate when MBP-a was expressed in E. coli. The trans-MBP-a-GroEL Complex as a Folding Competent Species—Due to the size constraint associated with the GroEL cavity, we suspected that during the folding reaction GroES can cap the MBP-a-GroEL complex only at the open end unoccupied by the large 86-kDa fusion polypeptide, resulting in the exclusive trans configuration. To address this question, we first studied the binding of a smaller polypeptide 48-kDa (His)6-protein A. The binding to the resin. The MBP-a moiety on the MBP-a fusion was unfolded and associated with GroEL, resulting in the formation of the MBP-a-GroEL complex. The MBP-a polypeptide was a preterminated translation product. The MBP-a-GroEL complex isolated from the E. coli lysate produced enzymatically active MBP-E1 when incubated with the β-GroEL complex and GroES-Mg2+ -ATP (data not shown). The result indicated that the MBP-a-GroEL complex was a productive folding intermediate when MBP-a was expressed in E. coli.
Fractions analyzed by SDS-PAGE, followed by Coomassie Blue staining. Resin extraction were separated on a 10–25% sucrose gradient, and proteins eluted with 20 mM maltose were subsequently separated on a 10–30% sucrose density gradient. The bottom of the gradient (fraction 15) contained 2 M sucrose. MBP-α* was a preterminated translation product.

When incubated with β-GroEL and 5 mM Mg²⁺-ATP, resulted in a full recovery of E1 activity, which served as a 100% control (Fig. 8B, lane 1). In the presence of PK but absence of nucleotides, GroES was incapable of binding to GroEL. (His)₆-α bound to GroEL was not protected and mostly digested (Fig. 8A, lane 2), which was consistent with the complete failure of the digested (His)₆-α to reconstitute E1 activity with β-GroEL (Fig. 8B, lane 2). In the presence of GroES, ADP, and PK, GroES was able to cap the (His)₆-α-GroEL complex in either trans or cis configuration (31). About half of (His)₆-α sequestered under GroES in cis was protected from PK digestion (Fig. 8A, lane 3). The proteolytically protected (His)₆-α in the presence of GroES and ADP was able to refold with the added β-GroEL complex and Mg²⁺-ATP, resulting in 42% of the control E1 activity (Fig. 8B). The data depict equal probabilities for GroEL to form cis and trans complexes with GroES and a small polypeptide. When GroES, 10 mM ATP, and PK were present, the (His)₆-α-GroEL complex underwent multiple rounds of polypeptide release and re-binding (40, 41). The dynamic alternations between cis and trans configuration eventually led to digestion of most of the (His)₆-α polypeptide through binding to GroEL in trans (Fig. 8A, lane 4). This result was supported by the absence of reconstituted E1 activity when the digested mixture was incubated with β-GroEL and Mg²⁺-ATP (Fig. 8B, lane 4).

In a parallel experiment, the MBP-α GroEL complex was incubated with GroES in the presence or absence of nucleotides and subjected to PK digestion. E1 activity reconstituted with undigested MBP-α-GroEL complex (lane 1) in the absence of nucleotides and PK (Fig. 9A, upper panel) and the β-GroEL complex was expressed as 100% (Fig. 9B, lane 1). In the absence of nucleotides but with (Fig. 9A, upper panel, lane 3) or without GroES (lane 2), MBP-α in the MBP-α-GroEL complex was completely digested by PK, with the MBP moiety remaining intact. Similar results was obtained when the MBP-

Due to space limitations, except for the 0-min point, only the MBP-α portion of the gel is shown. To normalize variations in Coomassie Blue staining, 1.5 μg of MBP-E1 was included in each gel (the Std. lane). The molecular mass markers (in kDa) used for calibration were: 40, MBP; 67, bovine serum albumin; 110, human E3; 158, aldolase; 232, catalase; 800, GroEL. B, the time course of folded MBP-α. The dye intensities of MBP-α bands in fractions 3–8 that contained MBP-α monomers, MBP-αβ dimers, and (MBP-α)₆ tetramers were measured by densitometry and summed to represent folding of the α moiety. The signal was linear with up to 10 μg of MBP-E1. The highest amount of MBP-α in combined fractions at the 4-h time point was expressed as 100%. C, time course for transition from dimers (fractions 4 and 5) (●) to tetramers (fractions 7 and 8) (○) in panel A and the recovery of E1 activity (○) from a separate experiment (Fig. 2, 0.1 μM) during the refolding.
α-GroEL complex was incubated with GroES and PK in the presence of ADP (lane 4) or ATP (lane 5). The complete digestion of MBP-α by PK was consistent with the absence of reconstituted E1 activity when the digested mixture was incubated with β-GroEL and Mg\(^{2+}\)-ATP (Fig. 9B, lanes 3–5). The residual E1 activity (Fig. 9B, lanes 2–5) represented background of the radiochemical assay. The capping of GroEL by GroES was examined by loading a fraction of the digestion mixture on the gel as shown in the Fig. 9A (bottom panel). The C-terminal 16 amino acid residues of the GroEL ring not capped by GroES were digested by PK, resulting in smaller cut monomers without affecting the GroEL function (31). The 1:1 ratio of uncut and cut GroEL (lane 4) confirmed that GroEL-GroES formed the 1:1 asymmetric complex in the presence of ADP as established by x-ray crystallographic studies (30). These data, taken together, establish that the formation of cis MBP-α-GroEL complex is sterically impossible, supporting the thesis that refolding of MBP-α is mediated exclusively through the trans configuration of the chaperonin complex.

GroES/Mg\(^{2+}\)-ATP-dependent Release of MBP-α Monomers from the MBP-α-GroEL Complex—To determine whether GroES was involved in the folding of MBP-α, the release and folding of the fusion polypeptide from the MBP-α-GroEL complex was studied. The MBP-α-GroEL complex was incubated with Mg\(^{2+}\)-ATP alone or Mg\(^{2+}\)-ATP and GroES for different durations. After incubation, MBP-α monomers separated on a sucrose density gradient were quantified by SDS-PAGE and densitometric scanning. Fig. 10 shows that, in the presence of GroES and Mg\(^{2+}\)-ATP, the MBP-α fusion is readily released from the MBP-α-GroEL complex, and exists as soluble monomers. The level of the released monomers remains constant from 10 min to 2 h. In contrast, when the complex is incubated with Mg\(^{2+}\)-ATP alone, the level of the discharged MBP-α monomers is less than 1/25 of that obtained when the complex is incubated with GroES and Mg\(^{2+}\)-ATP for the same durations. The results indicate that GroES is required for the efficient release and folding of MBP-α monomers from the MBP-α-GroEL complex.

**DISCUSSION**

Reconstitution of MBP-E1 with GroEL/GroES/Mg\(^{2+}\)-ATP in vitro provides a novel system to study the question of differential domain folding in the presence of chaperonins. The kinetic data from this study indicate that the refolding of MBP-E1 begins with the proper local folding of MBP and α moieties on the MBP-α chimeric polypeptide. This is ensued by the association of MBP-α (with the α moiety folded) and β into a dimer, and subsequent dimerization of two (MBP-α)β dimers into an (MBP-α)\(_2\)β\(_2\) tetramer. The time course for reconstitution of E1 activity essentially superimposes with that for the formation of MBP-E1 tetramers, suggesting that the dimerization step is rate-limiting in the renaturation of E1. This result is similar to the refolding of (His)\(_6\)-tagged E1 (14). The transition of dimers to tetramers as the rate-limiting step is also observed in homotetrameric proteins, e.g. bacterial phosphofructokinase (42) and lactate dehydrogenase, although their rates of folding are approximately 100-fold faster than that of E1 dimers. In the case of mitochondrial malic dehydrogenase, the rate constant for the association of two monomers into a native dimer is 3 × 10\(^4\) M\(^{-1}\) s\(^{-1}\) (45), which is 2 orders of magnitude higher than that of E1 refolding with rate constants of 376 M\(^{-1}\) s\(^{-1}\) for MBP-E1 and 465 M\(^{-1}\) s\(^{-1}\) for untaged E1. Moreover, malate dehydrogenase can refold at the same rate either in the presence or absence of GroEL/GroES, although the yield of spontaneous refolding is only 15%, which is considerably lower than that of GroEL/GroES-dependent folding (80–90%) of the same enzyme (45). In contrast, the folding of E1 has an absolute requirement for the presence of GroEL/GroES/Mg\(^{2+}\)-ATP, and an excess molar ratio (GroEL:E1 monomer) of GroEL is necessary to achieve the maximal degree of refolding (14).

MBP has been shown to function as a chaperone, similar to Hsp70 proteins, in assisting the refolding of citrate synthase. In the presence of DnaK (the bacterial homologue of Hsp70) or MBP, 22 and 13%, respectively, of refolding for citrate synthase can be achieved (44). Although less efficient than GroEL and GroES as chaperones (45), MBP or Hsp70 may help the refolding of citrate synthase by sequestering hydrophobic surfaces on the unfolded polypeptide. These results imply that the
periplasmic MBP may interact with unfolded proteins in that compartment, resulting in an increase in productive folding. In the present study, fusion of MBP moiety to the α sequence does not promote folding of the MBP-E1 proteins. In the MBP-α fusion, the rate constant for refolding of the MBP moiety (1.1 × 10^{-3} s^{-1}) is 20-fold slower than that measured with unfused MBP (0.020 s^{-1}), suggesting that there is intramolecular interference between MBP and α sequences during refolding of the fusion polypeptide. This observation is similar to the folding kinetics of individual domains during the spontaneous folding of multidomain proteins. The refolding of an individual domain is usually more rapid than when it occurs in a multidomain protein (21–25). The intermolecular interference in folding is of functional significance during protein transport. For example, the precursors of MBP (18) and ribulose-binding protein (46), the presence of leader sequences at N termini of these proteins reduces the rate of folding by 30–40-fold in comparison with their mature forms. It is proposed that the targeting sequence of some exported proteins could behave as an inhibitor of folding. The retarded folding of an exporting protein allows a sufficient time for the partially folded precursor to be transported to a correct target site in the cell with the help of Hsp70 (46). On the other hand, negative interactions between concurrently folding domains may result in intramolecular misfolding. As a case in point, only 10% of total Ras-dihydrofolate reductase was in the soluble native form when expressed in E. coli (26). It has been suggested that co-translational domain folding has evolved in eukaryotic cytosol to mitigate the adverse intramolecular interference during the folding of large multidomain polypeptides (26). However, this mechanism is not relevant to bacterial or mitochondrial proteins which fold post-translational or after import and processing (11–13).

Molecular chaperones may play the role of an unfoldase by reversing the off-pathway or that of a foldase by decreasing the activation energy of a folding intermediate along the folding pathway, and this results in an increase in the yield and/or rate of a folding reaction (6, 47–49). The similar rates for refolding of the MBP moiety on MBP-α in the absence and presence of GroEL/GroES indicate that GroE and GroES do not act as a catalyst for MBP folding. It is noteworthy, however, the yield of MBP refolding in the present study increases from 25 to 30% in spontaneous refolding to 70–90% when assisted by GroEL/GroES. In contrast, folding of the α moiety (rate constant, 2.95 × 10^{-4} s^{-1}) absolutely requires the presence of GroEL/GroES, and is about 1 order of magnitude slower than folding of the MBP moiety (rate constant, 1.9 × 10^{-3} s^{-1}) on MBP-α. The differential requirement for chaperonin-assisted folding remains unchanged for MBP and α moieties either as individual proteins or in the form of a fusion polypeptide. Moreover, refolding of MBP-α as assisted by GroEL and GroES produces an intermediate, i.e. the MBP-α-GroEL complex, in which part of the polypeptide chain, i.e. the MBP moiety, has already reached a native conformation which is resistant to PK and capable of binding to amylose resin, while the other segment of the chain, i.e. the α moiety, is still unfolded and associated with GroEL. This binding topology is expected to also occur in mitochondria between Hsp60 and large mature polypeptides with different folding characteristics between domains, as a means to circumvent aggregation.

The interaction between GroEL and GroES in mediating the folding of large polypeptides such as MBP-α is unknown. Studies on chaperonin-assisted refolding of small polypeptides, e.g.
ornithine transcarbamylase monomers (36 kDa), have shown that productive folding occurs exclusively through the cis complex, in which GroES and the unfolded polypeptide bind to the same ring of GroEL (31). However, this mechanism raises the question as to the size constraint for a polypeptide whose folding can be assisted by GroEL in a GroES-dependent manner. It has been speculated that GroES may play a role in promoting the productive release of large polypeptides from a trans configuration, that is, GroES and unfolded polypeptide bind to the opposite rings of GroEL (31). Here, PK digestion data provide evidence that the large MBP-α fusion polypeptide indeed binds to the asymmetric GroEL-GroES complex exclusively in trans (Fig. 9). One can argue that the MBP-α fusion forms a stable complex with GroEL but its folding is not assisted by GroES, similar to that observed with the large 124-kDa phytochrome photoreceptor (50) and the 72-kDa tail-spike protein of phage P22 (51). However, the level of MBP-α monomers released from the MBP-α-GroEL complex with GroES and Mg\(^{2+}\)-ATP is sharply higher than that obtained with Mg\(^{2+}\)-ATP alone (Fig. 10). Thus, our data strongly suggest that interactions between GroEL and GroES are needed for efficient folding of the MBP-α fusion. The availability of the stable MBP-α-GroEL binary complex in vitro provides a system to investigate how GroES promotes the productive release of large polypeptides from GroEL.

REFERENCES

1. Ellis, R. J. (1991) Annu. Rev. Biochem. 60, 321–347
2. Bukau, B., and Horwich, A. L. (1998) Cell 92, 351–366
3. Hartl, F. U. (1996) Nature 381, 571–580
4. Ellis, J. (1992) Nature 356, 191–192
5. Kim, S., Willison, K. R., and Horwich, A. L. (1994) Trends Biochem. Sci. 19, 543–548
6. Ostermann, J., Horwich, A. L., Neupert, W., and Hartl, F. U. (1989) Nature 341, 125–130
7. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilby, K., Dennis, D. T., Geeropoulous, C. P., Hendrix, R. W., and Ellis, R. J. (1988) Nature 333, 330–334
8. Waldinger, D. C., Eckerskorn, C., Lettspeich, F., and Cleve, H. (1988) Biol. Chem. Hoppe-Seyler 369, 1185–1189
9. Hartman, D. J., Hogenraad, N. J., Condron, R., and Hoj, P. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3394–3398
10. Legname, G., Fossati, G., Gromo, G., Monzini, N., Marcucci, F., and Modena, D. (1995) FEBS Lett. 361, 211–214
11. Stuart, R. A., Cyv, D. M., Craig, E. A., and Neupert, W. (1994) Trends Biochem. Sci. 19, 87–92
12. Schwarcz, E., and Neupert, W. (1994) Biochim. Biophys. Acta 1187, 270–274
13. Hartl, F. U. (1991) Semin. Immunol. 3, 5–16
14. Huang, J. L., Wynn, R. M., Song, J.-L., and Chuang, D. T. (1999) J. Biol. Chem. 274, 10395–10404
15. Wynn, R. M., Davie, J. R., Wang, Z., Cox, R. P., and Chuang, D. T. (1994) Biochemistry 33, 8962–8968
16. Wynn, R. M., Davie, J. R., Cox, R. P., and Chuang, D. T. (1992) J. Biol. Chem. 267, 12400–12403
17. Yeaman, S. J. (1989) Biochem. J. 257, 625–632
18. Chun, S.-Y., Strobel, S., Bassford, P. J., Jr., and Randall, L. L. (1993) J. Biol. Chem. 268, 20885–20882
19. Davie, J. R., Wynn, R. M., Cox, R. P., and Chuang, D. T. (1992) J. Biol. Chem. 267, 16601–16606
20. Wettlaufer, D. B. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 697–701
21. Blond, S., and Goldberg, M. E. (1986) Proteins 1, 247–255
22. Blond-Elguindi, S., and Goldberg, M. E. (1986) Biochemistry 25, 2409–2417
23. Frieden, C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4415–4416
24. Garel, J.-R., and Daugars-Varsat, A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3379–3383
25. Daugars-Varsat, A., and Garel, J.-R. (1981) Biochemistry 20, 1396–1401
26. Netzer, W. J., and Hartl, F. U. (1997) Nature 388, 343–349
27. Anfinsen, C. B. (1973) Science 181, 223–230
28. Braig, K., Owinskiwicz, Z., Hede, R., Boiervert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
29. Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, G. S., Ranson, N. A., Clarke, A. R., and Saubil, H. R. (1994) Nature 371, 261–264
30. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) Nature 388, 741–750
31. Weissman, J. S., Hoh, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., and Saffit, H. R., Fenton, W. A., and Horwich, A. L. (1995) Cell 83, 577–587
32. Goloubi, G., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44–47
33. Wynn, R. M., Davie, J. R., Chuang, J. L., Cote, C. D., and Chuang, D. T. (1998) J. Biol. Chem. 273, 13110–13118
34. Ferentz, T., and Lotz, U. (1978) FEBS Lett. 49, 213–217
35. Sambrook, J., Fritschi, F. E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
36. Voit, D., and Voet, J. G. (1990) Biochemistry, Wiley, New York
37. Sinclair, J. F., Ziegler, M. M., and Baldwin, T. O. (1994) Nat. Struct. Biol. 1, 320–326
38. Mendoza, J. A., Demeler, B., and Horowitz, P. M. (1994) J. Biol. Chem. 269, 2447–2451
39. Sparrer, H., Lilie, H., and Buchner, J. (1996) J. Mol. Biol. 258, 74–87
40. Martin, J., Mayhew, M., Langer, T., and Hartl, F. U. (1993) Nature 366, 226–233
41. Weissman, J. S., Kashi, Y., Fenton, W. A., and Horwich, A. L. (1994) Cell 78, 693–702
42. Deville-Bonne, D., Bras, G. L., Teschner, W., and Garel, J.-R. (1988) Biochemistry 27, 1217–1222
43. Jaenicke, R., Rudolph, R., and Heider, I. (1979) Biochemistry 18, 741–750
44. Richarme, G., and Caldas, T. D. (1997) J. Biol. Chem. 272, 15607–15612
45. Zhu, W., Landry, S. J., Giersch, L. M., and Squire, P. A. (1992) Protein Sci. 1, 522–529
46. Park, S., Liu, G., Toppino, T. B., Cover, W. H., and Randall, L. L. (1988) Science 239, 1033–1035
47. Rothman, J. E. (1988) Cell 59, 591–601
48. Martin, J., Langer, T., Botev, S., Kober, A., Horwich, A. L., and Hartl, F. U. (1991) Nature 352, 36–42
49. Flynn, G. C., Chappell, T. G., and Rothman, J. E. (1989) Science 245, 385–390
50. Grimm, R., Donaldson, G. K., van der Vies, S. M., Scharf, E., and Gatenby, A. A. (1993) J. Biol. Chem. 268, 5220–5226
51. Gordon, C. L., Sather, S. K., Casjens, S., and King, J. (1994) J. Biol. Chem. 269, 27941–27951