Inactive GroEL Monomers Can Be Isolated and Reassembled to Functional Tetradecamers That Contain Few Bound Peptides*

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For the first time, it has been shown that GroEL can be converted from tetradecamers (14-mers) to monomers under conditions commonly used for the preparation of this chaperonin. The essential requirements are the simultaneous presence of nucleotides such as MgATP or MgADP and a solid-phase anion-exchange medium. The monomers that are formed are metastable in that they only reassemble to a small degree in the absence of additives. These results are in keeping with previous studies on high pressure dissociation that showed the separated monomers display conformational plasticity and can undergo conformational relaxation when relieved of the constraints of the quaternary structure in the oligomer (Gorovits, B., Raman, C. S., and Horowitz, P. M. (1995) J. Biol. Chem. 270, 2061–2066). The monomers display greatly enhanced hydrophobic exposure to the probe 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid, although they are not active in folding functions, and they are unable to form complexes with partially folded rhodanese. The monomers can be completely reassembled to 14-mers by incubation in 1 M ammonium sulfate. There is no evidence of intermediates in the reassembly process. Compared with the original oligomers, the reassembled 14-mers have (a) very low levels of polypeptide contaminants and tryptophan-like fluorescence, two problems that previously hampered spectroscopic studies of GroEL structure and function; (b) functional properties that are very similar to the original material; (c) considerably decreased hydrophobic exposure in the native state; and (d) a similar triggered exposure of hydrophobic surfaces after treatment with urea or spermidine. This study demonstrates that the quaternary structure of GroEL is more labile than previously thought. These results are consistent with suggestions that nucleotides can loosen subunit interactions and show that changes in quaternary structure can operate under conditions where GroEL function has been demonstrated.

Molecular chaperones are proteins that can assist protein folding, and GroEL (cpn60) from Escherichia coli is perhaps the best studied member of this class. The initial step in the folding process is the formation of a noncovalent complex between the GroEL 14-mer and a non-native, interactive form of the refolding protein. This complex formation prevents misfolding and/or aggregation that competes with the acquisition of native structure (1, 2). Although the most efficient release of properly folded target proteins from GroEL requires the use of the co-chaperonin GroES (cpn10) and MgATP, it has been demonstrated that folding can be influenced by GroEL alone (3). GroEL interacts with such a wide variety of proteins (4) that recognition is presumably not sequence-specific, but rather, it must require some consensus physicochemical characteristic of the target proteins.

The essential features of the structure of GroEL have been revealed by electron microscopy (5) and x-ray crystallography (6). GroEL is a tetradecamer (14-mer) of presumably identical 60-kDa subunits organized in two stacked seven-membered rings to form a cylinder with a central channel. The individual monomers within the oligomer are folded into three domains: equatorial, intermediate, and apical. The apical domains, comprising residues 191–376, collectively contribute to forming the opening of the central cylindrical channel, and they are suggested to contain flexible segments that, by mutational studies, have been proposed to be involved in polypeptide chain binding (7). It has been suggested that quaternary structural changes in the 14-mer are important for binding proteins and protecting hydrophobic surfaces from nonproductive interactions as well as for transmitting information for cooperative binding and release in processes involving GroES interactions (8).

GroEL monomers can be formed at moderate concentrations of urea (e.g. 2.5 M), and they remain folded and capable of rapid reassociation upon removal of the urea (8). High hydrostatic pressure (e.g. 1.75 kilobars) yields GroEL monomers that display conformational drift to a form that does not rapidly reassociate upon depressurization (9). It has also been reported that monomeric GroEL can be isolated from a thermophilic organism, Thermus thermophilus (10). Monomeric GroEL has also been produced by limited proteolysis of the 14-mer by thermolysin (11). Finally, mutation of GroEL near the N terminus (e.g. Lys-3 replaced by Glu) yielded GroEL that was extensively dissociated to monomers (12).

In this work, we report that it is possible to prepare monomeric forms of GroEL under native conditions. The procedure relies on the destabilization of the 14-mer by nucleotides (either MgATP or MgADP) and the disassembly of these weakened oligomers by adsorption onto ion-exchange resins. The resulting monomers appear to be metastable and inactive, although they can subsequently be reassembled to fully active 14-mers. Thus, the formation of GroEL monomers is easier than formerly suspected, and previous preparative procedures produced monomeric GroEL during intermediate stages (13). One practical advantage of the present work is that it demonstrates that disassembly/reaassembly of 14-mers permits release and separation of bound impurities. Thus, it is possible to produce, in a way related to function, GroEL with little detectable tryptophan fluorescence and barely detectable levels of...
contaminating peptides, two difficulties with GroEL that prevented previous detailed spectroscopic studies.

EXPERIMENTAL PROCEDURES

Materials—All reagents were analytical grade. 1,1'-Bis(4-aminophthalene-5,5'-disulfonic acid (bisANS)1 was from Molecular Probes Inc. (Junction City, OR).

Method I: Purification of GroEL Monomers and Reassembly into 14-Mers—The chaperonins GroE and GroS were purified from lysates of cells bearing the multipolymer plasmid pGroEL (14) or pNDS (15). The procedure was performed at 0–4 °C and was somewhat modified from that of Clarke and co-workers (13). Protein concentrations were determined by either the bicinchoninic acid assay (Pierce) or the Bradford dye binding assay (Bio-Rad) as specified by the manufacturer.

Cell cultures were harvested by centrifugation at 4 °C. The cell pellets were dissolved in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 0.1 mM DTT and loaded onto a DEAE-Sephacel column (2.5 × 30 cm) equilibrated with the same buffer. Fractions were monitored for purity by SDS-PAGE and nondenaturing PAGE. At this stage, all fractions contained monomers of GroE or GroEL that were stored at 4°C.

For subsequent purification of GroEL, the ammonium sulfate suspension was centrifuged at 18,000 × g for 20 min, and the resulting pellet was dissolved in a solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 0.5 mM DTT. This solution was loaded onto a Sephacryl S-400 column (2 × 100 cm) equilibrated with the same buffer. The fastest fractions as judged by SDS-PAGE were pooled, and ammonium sulfate was added to 60% saturation. After centrifugation, the protein pellet was dissolved in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT and dialyzed overnight against the same buffer. The dialyzed material was loaded onto a DEAE-Sephacel column (2.5 × 30 cm) equilibrated with dialysis buffer. After washing with dialysis buffer, protein was eluted with a 0–0.4 M NaCl gradient. GroES eluted at 0.2–0.3 M NaCl, and GroEL eluted at 0.3–0.4 M NaCl. Fractions enriched with the chaperonins as judged by SDS-PAGE were separately pooled, and ammonium sulfate was added to 60% saturation. The ammonium sulfate suspensions containing GroES or GroEL were stored at 4°C.

Disassembly and Reassembly of Active Tryptophan-free GroEL

Method II: Alternative Purification of GroEL—The chaperonins GroEL and GroS were purified from lysates of E. coli cells bearing the multipolymer plasmid pGroEL (14). This purification was a modified version of published protocols (19, 20) as described previously (14). After purification, the chaperonins were dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol and then made 10% (v/v) in glycerol, rapidly frozen in liquid nitrogen, and stored at −70°C.

Purification of Rhodanese—Rhodanese was prepared as described previously (21) and stored at −70°C as a crystalline suspension in 1.8 M ammonium sulfate. Rhodanese concentrations were determined using A280 nm = 1.75 (22) and a molecular mass of 33 kDa (23). Rhodanese activity was assayed using a colorimetric method based on the absorbance at 460 nm of the complex formed between the reaction product, thiocyanate, and ferric ion (22).

bisANS Fluorescence Measurements—The fluorescence of bisANS was determined using an SLM-AMINCO 500C fluorometer. Excitation was at 395 nm, except where noted in the figure legends. Fluorescence intensities were determined at 500 nm or at the maxima of the spectra, which were scanned from 400 to 600 nm.

Ultracentrifugation—Analytical ultracentrifugation was done according to the protocol described previously (8). Briefly, samples of GroEL (0.44 μM 14-mer) in 50 mM Tris-HCl, pH 7.8, were subjected to sedimentation velocity in a Beckman XL-A analytical ultracentrifuge. Data from the ultracentrifugation were analyzed by the method of van Holde and Wissehet (25) using the Ultrascan ultracentrifuge data collection and analysis program (Bedemler, Missoula, MT).

Gel Electrophoresis—Nondenaturing gel electrophoresis was performed by the method of Neuhoff et al. (26) using 6% polyacrylamide gels at constant 200 V for a 1-mm thick gel. Denaturing gel electrophoresis was performed using 12% polyacrylamide by the method of Laemmli (27). Isoelectric focusing gels were run using a pH 5–7 gradient by the method of Robertson et al. (28). As noted under “Results,” either 7% or 8% polyacrylamide was used. For convenience, 5% acrylamide, 37.5% glycerol, and 0.8% bisacrylamide were used.

Chaperonin-assisted Refolding—Chaperonin-assisted refolding was performed as described previously (29). Briefly, rhodanese was denatured in 8 M urea, 200 mM sodium phosphate buffer, pH 7.4, and 1 mM β-mercaptoethanol at a protein concentration of 400 μM. Unfolded rhodanese was diluted (100 μM final concentration) into 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM KCl, 20 mM β-mercaptoethanol, and 50 mM sodium thiosulfate. Spontaneous folding was monitored by the region of rhodanese activity in the standard assay. Arrest of spontaneous folding was monitored using the rhodanese assay after addition of GroEL to a concentration of 2.5 μM (protomer). For chaperonin-assisted refolding in the complete chaperonin system, the samples were supplemented with GroES at 2.5 μM and ATP at 2 mM.

ATPase Activity Measurements—ATPase activity was measured as described previously by colorimetrically determining the decrease in ATP level with time (8). The ATPase activity is given in moles of ATP hydrolyzed of GroEL (protomer)/minute.

RESULTS

Monomers of GroEL Can Be Generated Using MgATP in Conjunction with Anion Exchange—Fig. 1 shows native and SDS gels of fractions from the QAE-Sepharose chromatography of Method I described under “Experimental Procedures,” in which the buffers contained MgATP. All the GroEL-containing fractions that eluted from the QAE-Sepharose column migrated on the native gel as monomeric protein. This is shown for the peak fraction in the third lane of the nondenaturing gel panel of Fig. 1. For comparison, the first lane of that panel shows the electrophoretic behavior of GroEL prepared by Method II, which was demonstrated to be a 14-mer by analytical ultracentrifugation (data not shown). The second lane of that panel shows that a sample from Method I contained GroEL as a 14-mer before it was loaded onto the QAE-Sepharose column. Separate experiments demonstrated that neither the presence of MgATP alone, nor the components of the column buffer, nor ion exchange in the absence of nucleotide

1 The abbreviations used are: bisANS, 1,1'-bis(4-aminophthalene-5,5'-disulfonic acid); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
would lead to significant disassembly (data not shown). Using this procedure, it was also shown that MgADP also produced monomers, thus indicating that ATP hydrolysis was not required for the disassembly. In these experiments, only the combination of MgATP (or MgADP) and anion-exchange chromatography produced monomeric GroEL. Additionally, two separate plasmids, pGroEL/ES (14) and pNDS (13), were used for GroEL preparations by Method I, and they gave identical results. Thus, the results are not dependent on the particular plasmid used for the preparation.

Monomers of GroEL Are Metastable and Are Not Active in Chaperonin Function—Pooled monomers (0.2 mg/ml) stored at 4°C did not noticeably reassemble as judged by native gel electrophoresis and analytical ultracentrifugation, which showed the monomers to have an s20w of 2.5 S, consistent with previous reports (8). Monomers were tested for several functions that define the chaperonin activity of GroEL. The monomers were inactive in refolding rhodanese in the presence of GroES, MgATP, and K+; and they did not significantly arrest the spontaneous folding of rhodanese. Thus, the monomers could not form complexes with unfolded protein. The monomers produced here behaved more like the monomeric species produced by high pressure (9) in that they did not spontaneously reassemble when subjected to native gel electrophoresis, which contrasts with the behavior of monomers formed in 2.5 M urea, which rapidly form 14-mers when the urea is removed, e.g., during electrophoresis (30). Isoelectric focusing showed that the monomers and 14-mers had similar pI values of ~5.2. This is in keeping with previous observations that the 14-mer and the monomers formed by urea treatment have the same charge-to-mass ratio (30). Thus, charge alterations are not responsible for the conversion of 14-mers to monomers. The monomers displayed considerably less ATPase activity than the 14-mers. The reassembled 14-mers showed ATPase activity of 5.7 units/μg, which is similar to that reported previously (31), while the monomers showed about one-seventh of that ATPase activity (<0.8 units/μg).

Monomers of GroEL Can Be Reassembled to 14-mers—The three panels of Fig. 1 show that GroEL can be reassembled. Fig. 1 (right panel) demonstrates that monomers could be reassembled by adding ammonium sulfate to a final concentration of 1 M as described under “Experimental Procedures.” The slow addition of ammonium sulfate to 1 M did not lead to a visible precipitate. The first lane of the right panel of Fig. 1 shows the behavior of 4 μg of the GroEL 14-mers (a sample similar to that in the first lane of the left panel). The second lane of the right panel of Fig. 1 corresponds to 4 μg of GroEL that was reassembled to 14-mers but not subjected to Sephacryl S-400 chromatography. It is clear that the reassembly is complete. These monomers did not reassemble significantly with MgATP alone, but there was some small amount of reassembly (~5%) after 20 × concentration of GroEL to 10 mg/ml with a Centricon 30 ultrafiltration unit at 4°C. If the ammonium sulfate was added quickly, a visible precipitate rapidly formed that, when dissolved and analyzed by ultracentrifugation, showed a mixture of monomers and 14-mers without any intermediate species. Fig. 1 also demonstrates that the monomers stain more strongly with Coomassie Blue than an equivalent amount of 14-mers, a result that is commonly observed (32).

Reassembled 14-mers Have Reduced Content of Low Molecular Mass Fragments—Previous studies indicated that almost all preparations of GroEL contained, in addition to the major 60-kDa band on SDS gels, substantial amounts of impurities that were isolated because they were bound to GroEL (33). Fig. 2 compares samples (8 μg of protein) from Method II (center lane) with the samples from Method I (right lane) that have been subjected to SDS-PAGE followed by silver staining. The level of contamination visible on silver staining of material from Method II is typical of many previous procedures (33). The content of contaminating species was considerably reduced by using the disassembly/reassembly protocol of Method I, and even the small amounts of low molecular mass peptides that are present in GroEL are largely removed by Sephacryl S-400 chromatography of the pooled ion-exchange fractions, as described under “Experimental Procedures.”
Reassembled 14-mers Have Very Low Tryptophan Contamination—Most previous preparations of GroEL had contamination from tryptophan-like fluorescence when excited at 280 nm (33, 34). This is unexpected since there are no tryptophans in the sequence of GroEL. Fig. 3 shows fluorescence spectra of samples of 1 μM GroEL from Method I (upper curve at 300 nm) and Method II (middle curve at 300 nm) compared with 0.4 μM N-acetyltryptophanamide. GroEL from Method I shows no indication of tryptophan fluorescence, and the spectrum is typical of proteins containing only tyrosine (35). Specifically, there is no maximum evident in the region between 335 and 355 nm where tryptophans emit. The difference spectrum between the two GroEL species has a λ<sub>max</sub> of ~345 nm (data not shown), consistent with intermediate exposure of the fluorescing tryptophans to solvent. A comparison of the integrated intensities under that tryptophan-like difference spectrum with the spectrum of the N-acetyltryptophanamide gives an estimate of 0.28 mol of Trp/GroEL monomer as the difference between the two preparations. These results were confirmed by determining the areas under fluorescence spectra for samples that were treated with 8 M urea and excited at 295 nm to normalize the Trp fluorescence, respectively. GroEL from Method II had an apparent content of 0.27 mol of Trp/GroEL monomer, while samples from Method I contained <0.025 mol of Trp/GroEL. Thus, the preparation that includes disassembly and reassembly (Method I) leads to the production of GroEL 14-mers that contain little or no tryptophan contamination.

Reassembled GroEL Regains Its Functional Properties—After reassembly, the GroEL 14-mers were tested for several functional activities. The ATPase activity of the 14-mers (5.5 units/μg) returned to a level commensurate with native GroEL (5.7 units/μg). The reassembled 14-mers were able to completely suppress the spontaneous folding of rhodanese. Reassembled GroEL had an efficiency for assisting the refolding of rhodanese in the complete chaperonin system that was comparable to protein that had not been disassembled, e.g. 75–80% recovery of rhodanese activity with GroES and ATP, based on a similarly treated control sample of rhodanese that was incubated but not denatured. These recoveries are within the range of values reported from previous studies of GroEL that was considered to be fully competent (29).

Urea-Induced Hydrophobic Exposure of Reassembled GroEL Is Facilitated by ATP or by Spermidine—GroEL has been suggested to operate, at least in part, by the controlled exposure of hydrophobic surfaces that are used to bind hydrophobic regions on target proteins, thereby suppressing the aggregation that competes with folding (36, 37). Hydrophobic exposure in GroEL was monitored by the binding of the hydrophobic fluorescent probe bisANS, and the results are shown in Fig. 4 for GroEL samples prepared by Method I or II. In Fig. 4A, the bisANS fluorescence (squares) with GroEL from Method II showed a biphasic response of hydrophobic exposure with increasing urea concentrations: one transition of increasing fluorescence intensity between ~2 and 3 M urea and a second transition of decreasing fluorescence intensity as the urea concentration was increased further. This behavior leads to a maximum fluorescence intensity between 2.5 and 3 M urea. The increasing exposure was previously demonstrated to accompany dissociation of the 14-mers into compact monomers, while the decreasing phase was suggested to result from their unfolding. The compact monomers could reassociate on dilution of the urea, but the unfolded monomers could not refold or reassociate properly to form the native 14-mers (30). The curve marked by triangles shows the same experiment for GroEL that was reassembled from monomers in Method I. This form of the protein exhibited similar behavior as described above. The monomers formed over approximately the same urea concentrations, and there was, at most, a slight shift in the denaturation part of the profile to higher urea concentrations. The major difference was observed at low urea concentrations, where there was
considerably less hydrophobic exposure in the reassembled 14-mer from Method I in the absence of any urea. This low initial hydrophobic exposure with GroEL from Method I apparently reflects the absence of bound, partially folded polypeptide chains that display hydrophobic exposure in GroEL from Method II.

It has been demonstrated that the presence of nucleotides shifts these bisANS transitions to lower urea concentrations, and as demonstrated in Fig. 4A, this also occurs with reassembled GroEL (24). It is interesting that the initial intensities of the two kinds of GroEL are much closer to each other at 0 M urea than they are in the absence of nucleotide. This is in keeping with the previously reported demonstration that GroEL can release bound polypeptide chains in the presence of ATP (33). Thus, reassembled GroEL has reduced binding of bisANS compared with GroEL that has never been disassembled, but it shows a urea-dependent increase in hydrophobic surfaces that is facilitated by ATP.

Fig. 4B shows the fluorescence spectrum of bisANS bound to 14-mers compared with the spectrum in the presence of an equivalent concentration of monomers. The lower curve is for the 14-mers that were reassembled from monomers, while the upper curve is for an equivalent amount of monomers before reassembly. The fluorescence quantum yield is enhanced by ~20-fold when the monomers are formed. This is even greater than the degree to which hydrophobic surfaces were enhanced when monomers were induced by high hydrostatic pressure (~10-fold) (9). Since these monomers do not extensively associate, as demonstrated by analytical ultracentrifugation, their hydrophobic surfaces, although accessible to the relatively small probe bisANS, are not sufficiently available for strong intermonomer interactions. When these monomers are treated with urea, there is no major increase in the bisANS fluorescence, and only the decreasing fluorescence is observed as the monomers denature, as observed in Fig. 4A.

Low concentrations of spermidine have been demonstrated to induce increased exposure of hydrophobic surfaces in the GroEL 14-mer, which was taken to indicate that GroEL hydrophobic exposure was responsive to polyvalent cations (24). Experiments were performed in the present work that showed that spermidine increased the hydrophobic exposure in the reassembled 14-mer in exactly the same way as was previously reported (data not shown). When 1 μM reassembled 14-mers was treated with 0–20 mM spermidine in the presence of 10 μM bisANS, the bisANS fluorescence exhibited a 4.0-fold enhancement of the fluorescence intensity with the half-maximum occurring at 2.2 mM spermidine. The results were very similar with GroEL prepared by Method II (maximum enhancement of 3.6-fold with the half-maximum occurring at 2.0 mM spermidine).

Intrinsic Fluorescence Can Follow Urea-induced Structural Transitions in GroEL—Previous studies of the denaturation of GroEL by urea using intrinsic fluorescence were based on samples that displayed significant tryptophan fluorescence (38). Thus, it was not clear whether the transitions that were observed reflected the unfolding of GroEL or whether they followed changes in the structure or binding of associated peptides. Fig. 5 shows that GroEL prepared by disassembly/reassemble in Method I displays a transition in the intrinsic fluorescence monitored at 310 nm (characteristic of the tyrosine emission maximum). The fluorescence intensity follows quite closely the dissociation and unfolding transitions that have been described previously, and the transition occurs in the same range of urea concentrations as the increasing fluorescence phase of the complex response in Fig. 4A (triangles).

**DISCUSSION**

These results provide a system with distinct advantages for studying some of the details of GroEL function. There are four fundamental results. 1) In the presence of MgATP or MgADP, ion-exchange chromatography leads to the dissociation of GroEL to metastable monomers that only slowly reassociate. 2) The monomers can be reassembled into competent 14-mers. 3) The reassembled 14-mers contain very low levels of peptide contaminants and tryptophan fluorescence, which have confounded previous studies of GroEL. 4) The subunit interactions are substantially altered by conditions required for GroEL function. It is interesting that the quaternary structure of the GroEL 14-mer, in the presence of nucleotides, is sufficiently delicate that it can be dissociated following ion-exchange adsorption. These monomers are similar to those formed under high pressure in that they appear to undergo conformational drift so they only slowly reassociate. This contrasts with monomers produced in 2.5 M urea, which rapidly reassemble after removal of the urea. Thus, 2.5 M urea apparently prevents those conformational changes that slow the reassociation of the monomers. Previous preparative procedures that used steps similar to those described here undoubtedly formed GroEL monomers at intermediate steps, but because SDS gels were used to gauge progress, it was not possible to assess the oligomeric structure of GroEL. Subsequent steps of those procedures, such as ammonium sulfate precipitation, would have led to reassembly (13).

These results support a model in which nucleotide binding loosens the quaternary structure, and since the oligomers presumably bind to the solid-phase exchanger via a limited number of subunits, they are effectively peeled apart. The conformational relaxation suggested to occur on the ion exchanger is similar to that observed with high pressure. Reassembly at increasing ionic strength may operate by loosening the structure of the metastable monomers in a manner similar to ion effects observed previously (24), and/or ions may electrostatically shield the negatively charged monomers, allowing them to interact and reassemble. Interestingly, if too much ammonium sulfate is added too quickly, rapid precipitation of monomers competes with reassembly, and the precipitated protein contains both monomers and 14-mers.

The disassembly/reassemble (Method I) helps eliminate two difficulties that have interfered with studies of GroEL. First, tryptophan-like fluorescence made it difficult to interpret experiments that sought to use the intrinsic fluorescence of GroEL to study structural changes. Second, bound polyptides influence the stability of the 14-mer, and the increased...
hydrophobic exposure that was ascribed to GroEL itself could be partly from these impurities. Also, heterogeneous bound polypeptides must give rise to a heterogeneous population of 14-mers in terms of physicochemical properties, thus complicating detailed analyses of structure-function correlations.

The results presented here show that the quaternary structure of GroEL is more delicate than has been previously appreciated, and this level of structure is influenced by ligands known to be functionally relevant. In addition, the conformation of the subunits are different when they are separated compared with their state(s) in the 14-mer. This type of conformational change could be envisioned as playing a role in the alternate exposure and burying of interactive surfaces on GroEL that are involved in engaging and releasing interactive surfaces on target proteins during GroEL function.

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