Monomer−Heptamer Equilibrium of the *Escherichia coli* Chaperonin GroES[†]

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ABSTRACT: In an effort to clarify the role of GroES in chaperonin-facilitated protein folding, a plasmid-encoding expression system for GroES incorporating a histidine-tagged, thrombin-cleavable, N-terminal sequence was constructed. This approach facilitated the rapid purification of native-like, histidine-cleaved GroES (HC-GroES). The addition of NaSCN to purification buffers to mildly promote subunit dissociation enabled the complete separation of chromosomally encoded, wild-type GroES chains from recombinant chains, allowing the production of homogeneous mutant variants of GroES. A substitution of histidine-7 to tryptophan in GroES was used to demonstrate the concentration-dependent modulation of the heptameric quaternary structure of the chaperonin. Fluorescence and light scattering studies of this mutant suggest that GroES heptamers dissociate to monomers upon dilution with half-times of 2−4 min. Sedimentation equilibrium experiments using either wild-type or HC-GroES can best be described by a monomer−heptamer equilibrium, yielding dissociation constants of 1×10^{-38} M^6 for native GroES and 2×10^{-32} M^6 for HC-GroES. These results are supported by subunit exchange experiments using mixtures of native or HC-GroES and GroES containing the complete N-terminal histidine tail. Native polyacrylamide gel electrophoresis demonstrates that these mixtures form an eight-membered hybrid set within minutes. The studies described here suggest a dynamic equilibrium for the quaternary structure of GroES, which may be an important feature for its role in GroEL-mediated protein folding reactions.

A wealth of evidence has accumulated recently on the role of the heat shock proteins GroEL and GroES from *Escherichia coli* in chaperonin-mediated protein folding (Hendrick & Hartl, 1993; Hartl & Martin, 1995). The importance of GroES in chaperonin-facilitated protein folding was first demonstrated in studies of the reconstitution of ribulose-bisphosphate carboxylase activity from denatured polypeptide chains (Goloubinoff *et al.*, 1989a). Numerous subsequent studies have shown convincingly that GroES is essential for GroEL-mediated protein folding reactions that are performed under conditions that disfavor spontaneous *in vitro* folding (Viitanen *et al.*, 1990; Martin *et al.*, 1991; Mendoza *et al.*, 1991; Zheng *et al.*, 1993). GroES binds strongly to GroEL in a nucleotide-dependent fashion (Viitanen *et al.*, 1990; Langer *et al.*, 1992; Jackson *et al.*, 1993; Todd *et al.*, 1993), possibly mediated by a flexible domain that becomes immobilized (Landry *et al.*, 1993) upon forming asymmetric (Langer *et al.*, 1992; Martin *et al.*, 1993; Chen *et al.*, 1994) or symmetric complexes (Azem *et al.*, 1994; Schmidt *et al.*, 1994b; Todd *et al.*, 1994). Possible roles for GroES have been suggested from evidence that the cochaperonin increases the cooperativity of ATP hydrolysis by GroEL to coordinate the release of polypeptide chains (Viitanen *et al.*, 1990; Todd *et al.*, 1993) or stabilizes an asymmetric chaperonin complex that is dissociated upon polypeptide chain binding (Martin *et al.*, 1993). However, it remains unclear why GroES is essential and how it facilitates the recovery of correctly folded proteins under "nonpermissive" refolding conditions (Schmidt *et al.*, 1994a).

In an effort to capitalize on the use of mutants of GroES for studies of its association with GroEL, a plasmid vector utilizing a histidine-tagged fusion was constructed. In addition, a rapid yet gentle method was developed to fractionate mutant variants of GroES and cleave the histidine-containing fusion peptide to yield native-like protein in the absence of detectable levels of wild-type contamination. The results presented here on wild-type and a tryptophan-containing mutant of GroES indicate that the chaperonin possesses a strong tendency to dissociate below micromolar concentrations. The relative ease of GroES dissociation raises a number of interesting questions regarding its role and should have important implications in its function with GroEL in chaperonin-facilitated protein folding.

EXPERIMENTAL PROCEDURES

Molecular Biological Procedures. Escherichia coli groES was cloned by amplification of pGroESL (Goloubinoff *et al.*, 1989b) via PCR techniques to introduce an *Nde*I restriction endonuclease site at the ATG initiation codon and a *Hin*dIII site at the TAA stop codon of the gene. This fragment was restricted and ligated into similarly digested pKK233-2 (Pharmacia) to yield pEGS1 for expression of wild-type GroES from the *trc* promoter upon induction with IPTG.[1]

A plasmid vector for the expression of His-tagged variants of GroES was constructed by first subcloning the *groES* gene into pET-15b (Novagen). Because the His-tagged GroES expressed from the T7 promoter with a 20 amino acid, $(His)_6$-containing, N-terminal fusion formed inclusion bodies, the *groES* gene with the sequence encoding the N-terminal His-

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containing peptide was subcloned in several steps into pKK233-2 to yield pEGSHis1. This construct, which results in the expression of GroES with the 20-residue segment M-G-S-S-$(H)_6$-S-S-G-L-V-P-R-G-S-H covalently linked to the usual N-terminal methionine, was then used for expression of His-tagged variants of GroES by IPTG induction from the *trc* promoter.

Amino acid substitutions in GroES were constructed in M13mp20 by oligonucleotide-directed mutagenesis using a kit (Amersham) containing phosphorothioate nucleotide analogs.

Cell Growth and Protein Expression. Expression of wild-type GroES was achieved in *E. coli* strain JV30 containing pEGS1 by addition of 1 mM IPTG at mid-log phase, and growth was continued for another 4−16 h. By contrast, JV30 containing pEGSHis1 was grown at 37 °C to late log phase ($A_{600} \sim 0.6-0.8$), at which time IPTG was added to 1 mM to induce the synthesis of His-tagged GroES from the *trc* promoter, and growth was continued for 2 h. Under these conditions, His-tagged GroES constitutes about 10% of the soluble protein in native cell lysates, with about half of the total GroES synthesized in an insoluble form.

Purification of Wild-Type and HC Variants of GroES. Wild-type GroES was prepared from JV30 containing pEGS1 through several steps. Cell lysates prepared by French press treatment were fractionated with a 35−55% ammonium sulfate cut, followed by DEAE Fast Flow chromatography in 50 mM Tris-HCl, pH 8.0, with elution by a linear gradient to 0.6 M KCl. Chromatography on Q Sepharose in 50 mM Tris-HCl, pH 7.0, was then employed with elution by a linear 0.4 M potassium phosphate gradient. This step was followed by S Sepharose chromatography in 20 mM MES, pH 6.0, eluted with a linear gradient to 0.2 M KCl. Final size exclusion chromatography on a Bio-Sil SEC-250 column (Bio-Rad) equilibrated in MES buffer resulted in pure fractions that were dialyzed against 50 mM Tris-HCl, pH 7.6, and stored either as an ammonium sulfate precipitate or made 10% with glycerol and stored at −80 °C. All buffers contained 0.1 mM DTT and EDTA. PMSF was present at 0.2 mM until the final step in the protocol.

Purification of His-tagged GroES and HC-GroES was achieved using nickel-conjugated His-bind resin (Novagen) essentially as suggested by the manufacturer with the following modifications. Cells were lysed with a French press, and prior to application of the crude lysate to the His-bind column, the extract was made 1 M in NaSCN to facilitate the separation of wild-type chains that had assembled into heptamers with His-tagged chains by dissociation of oligomers to folded monomers (Eisenstein & Schachman, 1989). The binding step and subsequent wash step were performed with buffers containing 1 M NaSCN, and upon elution, the fractions containing His-tagged GroES were immediately made 100 mM EDTA, which was found to minimize aggregation.

Cleavage of His-tagged GroES yielded His-cleaved GroES (HC-GroES) with 17 of the 20 N-terminal amino acids removed. Three residues (Gly-Ser-His) remain since they lie within the thrombin cleavage site of the His-tagged fusion sequence. Digestion was achieved by first dialyzing the protein against 20 mM Tris-HCl, pH 8.4, 150 mM NaCl, and 50 mM EDTA, followed by a change into the same buffer with a reduced (1 mM) level of EDTA. Cleavage of the histidine-containing peptide was accomplished using 10 units of thrombin/mg of His-tagged GroES (Haematologic Technologies, Essex Junction, VT) for 6 h at room temperature. The resulting mixture was dialyzed against wash buffer (Novagen) and reapplied to the His-bind column in order to bind and remove uncleaved chains and the histidine peptide. The column wash containing unbound HC-GroES was dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA and DTT for a final chromatography step employing POROS HQ50 with elution by a linear 0.5 M NaCl gradient.

Spectroscopic Measurements. Fluorescence measurements were performed using a FluoroMax 2000 spectrofluorometer from Spex Industries. Relative fluorescence emission of $GroES_{H7W}$ was measured between 0.3 and 10 μM monomer concentrations using an excitation wavelength of 295 nm with the excitation slit set at 0.25 mm and the emission slit set to 6 mm. Measurements of light scattering by GroES were made using the FluoroMax 2000 with the excitation and emission monochromaters set at 500 nm using 1-mm slit widths.

Sedimentation Equilibrium. Equilibrium sedimentation experiments using native and mutant variants of HC-GroES were performed with a Beckman XL-A Optima analytical ultracentrifuge and a four-hole, An-55 rotor. All experiments were performed at 25 °C at rotor speeds between 28 000 and 32 000 rpm. The concentration distribution of GroES at sedimentation equilibrium was usually acquired as an average of 25 measurements of absorbance at each radial position, with nominal spacing of 0.001 cm between radial positions. Samples were dialyzed extensively against 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and DTT, and solvent densities were determined pychnometrically. A partial specific volume of 0.736 mL/mg was assumed for GroES on the basis of its amino acid composition (Hemmingsen *et al.*, 1988).

Equilibrium sedimentation data of GroES samples obtained at either two different rotor speeds or two initial protein concentrations were analyzed for average molecular weights in terms of a single, homogeneous species as previously described (Eisenstein, 1994). Equilibrium constants for the dissociation of heptameric GroES to monomers were estimated from the data obtained at sedimentation equilibrium according to

$$c_r = B + c_{m,M} \exp[M_M(1 - \bar{v}\varrho)\omega^2(r^2 - r_m^2)/2RT] + (c_{m,M})^7/K_{71} \exp[M_H(1 - \bar{v}\varrho)\omega^2(r^2 - r_m^2)/2RT] \quad (1)$$

where c_r is the concentration of the protein at a given radial position, $c_{m,M}$ is the concentration of monomeric species at a reference position, M_M and M_H are the monomer and heptamer molecular weights, K_{71} represents [monomer]^7/[heptamer], $\bar{v}$ is the partial specific volume, ϱ is the solvent density, ω is the angular velocity, r is the radial position in centimeters from the center of rotation, r_m is the distance in centimeters from the center of rotation to the reference

[1] Abbreviations: His-tagged GroES, GroES synthesized with an N-terminal 20 amino acid peptide that contains a six histidine repeat and a thrombin cleavage site; HC-GroES, GroES expressed as a His-tagged fusion protein and subsequently cleaved with thrombin and fractionated from the His-tagged peptide; MES, 2-(*N*-morpholino)-ethanesulfonic acid; IPTG, isopropyl thio-β-galactoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

position (e.g., the meniscus), R is the gas constant, T is the absolute (Kelvin) temperature, and B is a correction term for a non-zero baseline. Parameters were evaluated using nonlinear least-squares analysis (Johnson *et al.*, 1981) or by using a modified version of IGOR (Wavemetrics, Lake Oswego, OR) running on a Macintosh computer (Brooks *et al.*, 1994).

Miscellaneous Analytical Methods. Electrophoresis experiments in native polyacrylamide gels were performed on a Pharmacia Phast System using 12.5% homogeneous gels. Tracer sedimentation equilibrium experiments were performed as suggested by Minton (Darawshe *et al.*, 1993) using a Brandel microfractionator (Attri & Minton, 1986). The association of native and HC variants of GroES with GroEL was measured using size exclusion chromatography essentially as described (Viitanen *et al.*, 1990). The effect of wild-type and HC-GroES variants on the GroEL-promoted refolding of rhodanese (Mendoza *et al.*, 1991) was measured using published protocols. Tritium labeling of wild-type GroES was achieved in either the absence or presence of GroEL (with nucleotides) by reductive methylation with sodium borotritide (Tack *et al.*, 1980).

RESULTS

Preparation of Native-like Variants Free from Wild-Type GroES. A strategy was devised to express plasmid-encoded, mutant variants of GroES that could be purified from chromosomally encoded wild-type chains expressed in the same cells. A functional *groES* gene is essential for *E. coli* growth at all temperatures (Fayet *et al.*, 1989). Consequently, when His-tagged GroES was expressed in a strain that possessed the wild-type *groES* gene, the resulting oligomers were found to contain up to 10% wild-type chains by polyacrylamide gel electrophoresis. The addition of a chaotropic agent was therefore employed to gently dissociate these oligomers for the separation of wild-type GroES. Buffers containing 1 M NaSCN enabled the specific binding of the His-tagged monomers to the Ni^{2+}-containing affinity resin, with the removal of contaminating wild-type chains in the wash buffer, and subsequent elution of a homogeneous His-tagged species. After cleavage of the His-tagged sequence with thrombin, the mixture was passed through the nickel column a second time in which HC-GroES eluted with the wash buffer. Further fractionation of HC-GroES by ion-exchange chromatography resulted in the preparation of GroES that displayed a single band on both native and SDS–polyacrylamide gel electrophoresis.

This protocol enabled the rapid and efficient production of 1–3 mg of HC-GroES/g of cells. The resulting histidine-cleaved (HC-) GroES, containing the three additional amino acids Gly-Ser-His adjacent to the N-terminal methionine, was found to function like wild-type, native GroES as evidenced by its strong association with GroEL during size exclusion chromatography in the presence of adenosine nucleotides (Viitanen *et al.*, 1990). In addition, not only HC-GroES but His-tagged GroES and the H7W mutant of GroES were equivalent in facilitating the GroEL-promoted refolding of rhodanese under permissive and nonpermissive conditions (data not shown) (Mendoza *et al.*, 1991).

Spectroscopic Evidence for Dissociation of $GroES_{H7W}$ upon Dilution. With the ability to prepare mutants of GroES and to purify them from wild-type chains, genetic variants of HC-GroES were sought for studies of its nucleotide-promoted binding to GroEL. However, initial results from these studies supported earlier observations (see below) which suggested that heptameric GroES dissociates to monomers at submicromolar concentrations. Preliminary evidence for the dissociation of GroES came from an examination of the spectroscopic properties of tryptophan-containing mutants of GroES. As can be seen in Figure 1, the dilution of $GroES_{H7W}$ results in a slow decrease in the fluorescence intensity at 346 nm, the wavelength of maximum emission. Analysis of this fluorescence change in terms of a single kinetic phase yields a half-time for the decrease of about 2 min. It is of interest that the decrease in fluorescence is paralleled by a decrease in the light scattering intensity seen for the same mutant. As can be seen in Figure 2, there is a decrease in the relative intensity of scattering by $GroEL_{H7W}$ that follows upon its dilution into buffer. Analysis in terms of a single exponential decrease yields a half-time of about 4 min, within a factor of 2 from that seen from fluorescence. Neither the presence of KCl, an effector for ATP and polypeptide chain binding to GroEL, nor the magnesium salts of ATP or ADP showed any effect on the spectroscopic properties of $GroES_{H7W}$.

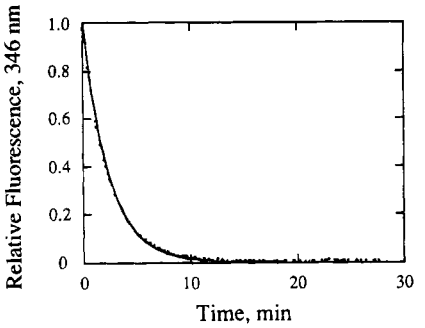

FIGURE 1: Time dependence of the fluorescence changes upon dilution of HC-$GroES_{H7W}$. The relative fluorescence at 346 nm versus time was measured upon dilution of a 175 μM (monomer) solution of HC-$GroES_{H7W}$ to a final concentration of 0.7 μM monomer in 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 mM KCl, and 0.1 mM DTT at 25 °C. The decrease in fluorescence can be described in a single kinetic phase with a half-time of approximately 1.6 min.

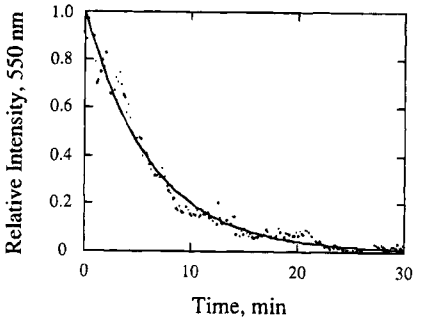

FIGURE 2: Time-dependent light scattering changes of HC-$GroES_{H7W}$. The decrease in light scattering at 500 nm versus time upon dilution of a 175 μM (monomer) solution of HC-$GroES_{H7W}$ to a final concentration of 7 μM was measured in 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 mM KCl, and 0.1 mM DTT at 25 °C. The decrease in scattering can be described by a single phase with a half-time of about 4.3 min.

Sedimentation Equilibrium. The spectroscopic results on the apparent dissociation of $GroES_{H7W}$ upon dilution were consistent with earlier efforts using ^{3}H-labeled GroES in tracer sedimentation equilibrium experiments to measure the

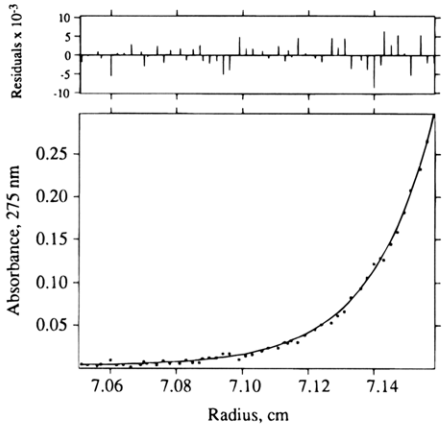

FIGURE 3: Sedimentation equilibrium of wild-type GroES. Sedimentation equilibrium was performed in 50 mM Tris-HCl, pH 7.5, in the presence of 0.1 mM EDTA and 0.1 mM DTT at 25 °C. (Bottom panel) Absorbance at 275 nm versus distance from the center of rotation in centimeters. (Top panel) The residuals ($A_{275nm,theoretical} - A_{275nm,obsd}$) for a monomer−heptamer model with an equilibrium constant of 1×10^{-38} M^6 are small and random.

energetics of its association with GroEL. Fractionation of ^{3}H-labeled GroES gradients achieved by sedimentation equilibrium experiments in the presence of density stabilizers at protein concentrations as low as 200 nM (monomer) yielded average molecular weights of only 10 000−20 000 (data not shown), significantly lower than the estimated value of 72 600 based on a heptameric structure of 10 370 Da chains.

Conventional sedimentation equilibrium was therefore employed to investigate the effect of concentration on the quaternary structure of GroES. Typical data for wild-type GroES are presented in Figure 3. These data, as well as additional data collected at different initial loading concentrations, were poorly described by a model for a single, homogeneous species and yielded average molecular weights of only 55 000−60 000, considerably lower than that expected for a heptameric oligomer. Alternatively, as can be seen in Figure 3, a much better fit to the data was obtained using eq 1, which describes a model for a monomer−heptamer equilibrium, yielding an equilibrium dissociation constant for wild-type GroES of 1×10^{-38} M^6.

Sedimentation equilibrium results of HC-GroES and the H7W variant, both of which contain the three additional amino acids at the N-terminus, were also described more precisely by the monomer−heptamer model and yielded similar, but slightly weaker values for the dissociation constant in the range of $2-4 \times 10^{-32}$ M^6.

Hybridization of Wild-Type and His-Tagged GroES. Further evidence to support the interpretation of the dissociation of GroES was obtained from subunit hybridization experiments. These experiments capitalized on the difference in electrophoretic mobility between wild-type GroES and the His-tagged variant to determine the conditions under which the dissociation and random reassociation of these two chains would yield a set of intersubunit hybrids. As can be seen in Figure 4, there is a considerable difference in the mobility on native polyacrylamide gels between wild-type or HC-GroES relative to the His-tagged variant that contains an additional 20 amino acids with 6 positively charged histidines. In addition, it is of interest to note the homogeneity of the His-tagged band, reflecting the absence of any wild-type chains in the preparation. When equimolar concentrations of His-tagged GroES are mixed with either wild-type or His-cleaved GroES in neutral buffer and then loaded on the polyacrylamide gel, an eight-membered hybrid set can be resolved. These experiments, performed at micromolar protein concentrations, reflect the dynamic equilibrium of GroES monomers with oligomers.

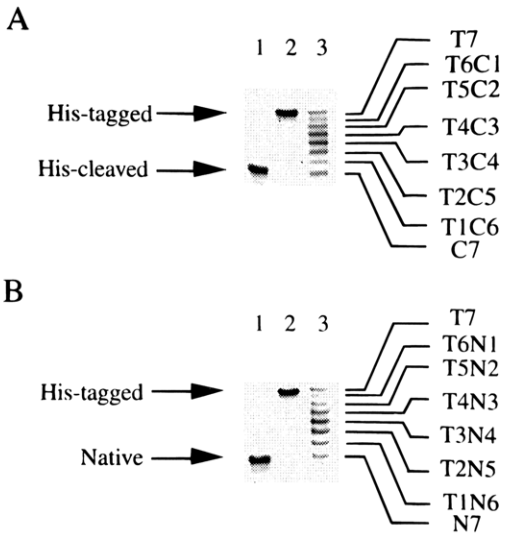

FIGURE 4: Dissociation of wild-type GroES and HC-GroES as shown by the formation of interchain hybrid heptamers. Mixtures of equal amounts of either wild-type GroES or HC-GroES with His-tagged GroES were made in 50 mM Tris-HCl, pH 7.6, at 25 °C, and were immediately analyzed by native polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. (A) Lane 1, 28 μM HC-GroES; lane 2, 28 μM His-tagged GroES, prior to cleavage of the 20-residue histidine-tagged N-terminal tail; lane 3, mixture of HC-GroES and His-tagged GroES. The generation of an eight-membered hybrid set is labeled as the parental species containing seven cleaved monomers (C7), seven His-tagged monomers (T7), and intermediate species. (B) Lane 1, native, wild-type GroES; lane 2, His-tagged GroES; lane 3, eight-membered hybrid set labeled as described above.

DISCUSSION

The spectroscopic, sedimentation, and hybridization experiments presented here reveal the tendency of heptameric GroES to dissociate to monomers at sub-micromolar monomer concentrations. This raises several interesting questions regarding the function of this component of the chaperonin system. The good fit of the monomer−heptamer model to sedimentation equilibrium data argues that the assembly of GroES occurs by a highly cooperative process; otherwise, stable intermediates would have been detected by sedimentation equilibrium.[2] This can be seen in Figure 5, which relates the theoretical fraction of monomer for wild-type and HC-GroES to the logarithm of the total (monomer) concentration. From this plot it can be estimated that, at equilibrium, wild-type GroES exists in a roughly equal mixture of monomers and heptamers at a total concentration of 6.7×10^{-7} M. Although the HC variant of GroES was also described by the monomer−heptamer equilibrium model, it was more

[2] A partially assembled intermediate of GroES would need to comprise more than 5% of the total material for detection with absorption optics by sedimentation equilibrium. However, various models that included dimeric, trimeric, or higher order oligomeric terms did not yield any improvement in the fits to the concentration distributions. No more than three oligomeric species were considered in any model due to constraints on parameter convergence.

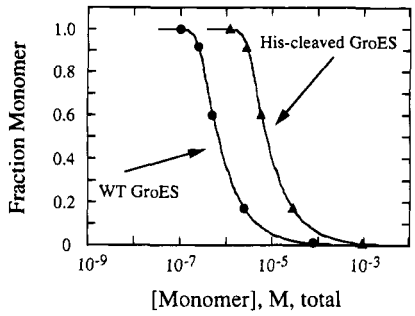

FIGURE 5: Equilibrium fractions of monomer for GroES and HC-GroES versus the logarithm of total monomer concentration. Theoretical curves were generated from dissociation constants of 1×10^{-38} M^6 for native GroES and 2×10^{-32} M^6 for HC-GroES. This yields an effective $C_{0.5}$ of approximately 6.7×10^{-7} M for wild-type GroES and a value of 7.5×10^{-6} M for HC-GroES.

weakly associated, leading to an elevated concentration of about 7.5×10^{-6} M monomer for an equimolar mixture. Thus, although both HC-GroES and native, wild-type GroES are functionally indistinguishable, the His-cleaved variant, containing three additional amino acids at its N-terminus, shows a slight increase in its relative ease to dissociate.

The half-life of the GroES heptamer may be only minutes as revealed by subunit exchange performed at micromolar concentrations. Complete exchange between wild-type and His-tagged GroES to yield an eight-membered hybrid set in a roughly binomial distribution took place within the time of mixing the two proteins and loading a polyacrylamide gel (~10−15 min). In addition, this estimate is in agreement with the fluorescence and light scattering measurements which yield half-times of between 2−4 min for heptamer dissociation, assuming that these techniques accurately reflect the dissociation of heptameric GroES upon dilution. The slight differences in the half-times estimated by these various methods may be attributed to the differences in the signals observed, since the extent of GroES dissociation should be controlled solely by the total concentration.

Several studies on the effect of ligands on the dissociation of GroEL (Horovitz *et al.*, 1993; Mendoza *et al.*, 1994; Mendoza & Horowitz, 1994) have suggested that changes in subunit interactions within the tetradecamer may underlie chaperonin function. In this regard, the presence or absence of nucleotides (as magnesium salts) or K^+ ions, ligands which display measurable effects on the subunit interactions of wild-type GroEL, did not show any significant effect on the apparent dissociation rate of GroES. Although photolabeling studies using azido-ATP have implicated a role for nucleotide binding to GroES (Martin *et al.*, 1993), the lack of their effect on the rate of heptamer dissociation could be consistent either with the apparent nonspecificity of azido-ATP cross-linking (Todd *et al.*, 1995) or with the possibility that nucleotides bind to GroES monomers, which would not alter the intrinsic rate of dissociation. It would therefore be of interest to identify other factors that promote an alteration of subunit interactions within GroES and affect its tendency to dissociate. In addition, the effect of ligands or mutations to either significantly enhance or inhibit GroES dissociation may shed light on its role in chaperonin-facilitated protein refolding.

A range of GroES concentrations above and below micromolar levels have been used in studies of the GroEL-promoted reconstitution of enzyme activities. In examples of reconstitution reactions performed at relatively high GroES concentrations where heptamers predominate (Fisher & Yuan, 1994; Schmidt *et al.*, 1994a; Weissman *et al.*, 1994), there is doubtless a specific, "coupling" effect of GroES on GroEL function. On the other hand, titration experiments to measure the stoichiometry of the effect of GroES on GroEL have been performed at relatively low concentrations (Holl-Neugebauer *et al.*, 1991; Langer *et al.*, 1992), where GroES is largely monomeric. It is possible, therefore, that some of the effect seen in experiments performed at sub-micromolar GroES concentration may be due simply to competition with non-native chains for binding to GroEL, thereby promoting their release into solution where they can refold. In an extensive mutagenesis study to correlate the functional roles of the structural domains of GroEL, most amino acid substitutions in the apical domain of GroEL that affect the ability of the chaperonin to bind non-native polypeptides also diminish the nucleotide-promoted association of GroES with GroEL (Braig *et al.*, 1994; Fenton *et al.*, 1994). If non-native polypeptide chains compete with GroES for a similar binding site on GroEL, and the monomers of GroES possess non-native character, then one could imagine a direct competition between these two effectors for GroEL.

Although the affinity of GroES for GroEL has been estimated in the nanomolar range (Jackson *et al.*, 1993; Todd *et al.*, 1993), it has been observed that GroES binds slowly (half-time ~2 min) to pyrene-labeled GroEL (Jackson *et al.*, 1993). At the relatively low (980 nM monomer) concentration of GroES used in these studies, it is possible that the binding kinetics reflect the slow reassociation of GroES monomers to oligomers, which then bind to GroEL.[3] Alternatively, GroES could assemble on the surface of GroEL. Because GroES displays detectable binding to GroEL only in the presence of adenosine nucleotides, further studies on the assembly of GroES, in the absence and presence of GroEL, will be required to resolve these two possibilities.

The development of an expression system and purification procedures that produce homogeneous variants of GroES, in the absence of contaminating wild-type protein, should facilitate studies of the effect of mutations on the functional properties of the chaperonin. Recently, the purification of GroES from mammalian mitochondria and *Bacillus stearothermophilus* has been reported using His-tagged fusions at the C- and N-termini, respectively (Dickson *et al.*, 1994; Schon & Schumann, 1994). However, neither of these variants was constructed with a protease cleavage site for removal of the His-tagged sequence after purification. It is of interest, however, that mitochondrial GroES, like His-tagged GroES prepared as described here, was fully functional in refolding experiments under nonpermissive conditions with bacterial GroEL, in spite of an uncleaved 13-residue C-terminal His tag.

In conclusion, an expression system and purification method have been developed to prepare mutants of the *E. coli* chaperonin GroES which are free of detectable levels of wild-type chains. These mutants, as well as their variants

[3] Concentration jump experiments on the assembly of GroES in the presence of a trace amount of a tryptophan-containing mutant suggests that the half-time for assembly of wild-type GroES at about 1 μM monomer concentration is approximately 2 min (J. Zondlo and E. Eisenstein, unpublished observations).

containing an N-terminal, histidine-containing extension, have been used in biophysical studies to probe the relative stability of the quaternary structure of GroES, revealing a dynamic equilibrium between monomers and heptamers. The reversible dissociation of GroES should have significant implications for future studies on its binding to GroEL and on its role in chaperonin-facilitated protein folding.

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