Ligand-induced Conformational Changes in the Apical Domain of the Chaperonin GroEL*

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Although the role of nucleotides in the catalytic cycle of the GroESL chaperonin system has been extensively studied, the molecular effects of nucleotides in modulating exposure of sites on GroEL has not been thoroughly investigated. We report here that nucleotides (ATP, ADP, or adenosine 5'-[β,γ-imino]triphosphate) in the presence of Mg²⁺ make the oligomer selectively sensitive to trypsin proteolysis in a fashion suggesting conformational changes in the monomers of one heptameric ring. The site of proteolysis in the monomer that is exposed upon nucleotide binding by the oligomer is in the apical domain (Arg-268). Further, complexes of GroEL with GroES or rhodanese display the same sensitivity to proteolysis, unlike the GroEL-GroES-rhodanese complex, which is protected from proteolysis. The influence of various cations on trypsin proteolysis is investigated to elucidate the differential effects that monovalent and divalent cations have on the oligomeric structure of the chaperonin. These results are discussed in relation to the molecular basis for the chaperonin activity.

The molecular chaperones are a class of proteins that have been shown to facilitate the in vivo folding and transport of nascent polypeptides (1, 2). The chaperonins, one class of molecular chaperones, have been found in prokaryotes, mitochondria, and chloroplasts (3). One widely studied chaperonin from Escherichia coli, GroEL, has been demonstrated to promote the in vitro refolding and assembly of a variety of chemically denatured proteins, including rhodanese (4, 5), ribulose-bisphosphate carboxylase/oxygenase (Rubisco) (6, 7), and glutamine synthetase (8). The promiscuity of GroEL in polypeptide recognition and binding suggests that the hydrophobic interactions accounting for the complex formation vary from substrate to substrate (9-11). The fact that some proteins require only K⁺ and ATP-Mg (or a non-hydrolyzable analog) for release of functional enzyme from the complex, whereas others also require the co-chaperonin GroES, supports this idea that complexes with some non-native proteins are stronger than others (12, 13).

GroEL is a homotetradecamer (14-mer) of 57.2-kDa subunits, arranged as two stacked heptameric rings, with a central cavity at each end (14). The 2.8-Å x-ray crystal structure reveals that each monomer is organized into an equatorial, intermediate, and apical domain, with interactions between equatorial domains exclusively defining the heptamer-heptamer interface (15). Monomer-monomer interactions within each ring are mediated by the equatorial domains and by conserved interactions at the intermediate-apical domain interface (15, 16). The apical domains line the opening to the central cavity at either end of the oligomer and contain a region (200-263) that has been implicated by site-directed mutagenesis and photointerception of the hydrophobic probe bis-ANS1 as the site of polypeptide binding (17, 18).

Studies of the effects of nucleotides on GroEL have focused on regulation of the ATPase activity of the chaperonin (19, 20), including the elements of positive and negative cooperativity in nucleotide binding/hydrolysis, and the debate over complex formation (21-26). Several studies have shown that two GroES molecules can bind to a single GroEL oligomer in the presence of ATP to form symmetric complexes, while others have demonstrated asymmetric complexes (1:1 GroEL:GroES ratio) to be the functional, physiological unit. The few studies that have evaluated the conformational changes in GroEL due to ATP binding or hydrolysis have demonstrated quaternary structural changes consisting of monomer pivoting and apical domain reorientation upon ATP binding (27-29). Unfortunately, the results do not provide much information about the exposure of specific sites believed to mediate protein-protein interactions. Finally, nucleotides have also been shown to affect the affinity of GroEL for substrate, with the ADP-Mg complex displaying weak binding (30).

There have been several reports on ions (monovalent, divalent, and polyanion) affecting structural changes in GroEL (31-33). Monovalent and polyanionic cations are reported to increase the exposure of hydrophobic surfaces on GroEL without disrupting the oligomeric structure (33). Divalent, but not monovalent, cations stimulate the ATPase activity of GroEL and induce structural changes that allow preferential cross-linking of the heptameric rings (31). These results suggest that cations of various charges may work at several different levels to produce structural changes in the GroEL oligomer.

In this report we show that proteolysis by trypsin can be used as a probe for conformational changes in the tetradecameric structure of GroEL. In a native state with Mg²⁺ as the only ligand, GroEL is not susceptible to proteolysis by trypsin, whereas unliganded GroEL is rapidly digested, leaving only 10% of intact monomers within 30 min. Upon addition of nucleotides (ADP, ATP, or the ATP analog AMP-PNP) and Mg²⁺, exposure of one predominant cleavage site in the apical domain occurs. When ADP-Mg is liganded to GroEL, producing a state with high affinity for non-native protein, approximately half of the monomers become proteolyzed without loss of the oligomeric structure. Complexes of GroEL-ADP-Mg with GroES or denatured rhodanese are proteolytically sensitive in a manner.

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1 The abbreviations used are: bis-ANS, 4,4'-bis[1-anilino-8-naphthalenesulfonic acid]; AMP-PNP, adenosine 5'-[β,γ-imino]triphosphate.
similar to the nucleotide-ligated state. In contrast, a GroES-GroEL-rhodanese complex formed in the presence of ADP-Mg is significantly protected from proteolysis (~90% protected). Monovalent and polyvalent cations also affect trypsin proteolysis of GroEL, protecting approximately half of the sites from cleavage compared with an unliganded state. These results suggest that nucleotide binding induces asymmetry in the GroEL tetradecamer, exposing specific sites in the apical domain of only one heptameric ring such that they are accessible to other proteins in solution, either protease or substrate proteins.

MATERIALS AND METHODS

Reagents and Proteins—All reagents used were of analytical grade. Trypsin was 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated type XII purchased from Sigma. Low molecular weight markers were purchased from Bio-Rad. Hide powder azure was purchased from PolyScience. Amino acid and protein sequence analyzer (Applied Biosystems, Foster City, CA). Peptides were subjected to approximately 12 rounds of sequencing. The sequences of the fragments were compared with the published sequence of GroEL to determine their positions within the intact protein (3).

Scanning and Quantitation of Protein Gels—Coomasie-stained gels were imaged using a CCD 505 video camera (CCTV Corp., New York) under the control of NIH-Image software running on a Macintosh II/1 microcomputer. Band densities were quantified using the densitometry options of the software.

High Performance Liquid Chromatography—Samples were chromatographed on a TSK-G4000PW (30 × 7.5 mm) column equilibrated with 25 mM NaPO4, 50 mM NaCl, 0.1 mM EDTA, pH 7.6, and monitored at 280 nm.

Curve Fitting—Except where noted in the figure legends, data were fit using a non-linear least squares fitting procedure implemented in the software program, PSI Plot (Poly Software International, Salt Lake City, UT). Where data were fit to an exponential, the following equation was used: \( y = A_1 e^{-kt} + A_2 \), where \( y \) represents the measured value, \( t \) represents time in minutes, \( A_1 \) represents the fraction of total protein susceptible to digestion (\( A_1 e^{-kt} \) therefore represents the fraction of protein digested at time \( t \)), and \( A_2 \) represents the fraction of protein resistant to proteolysis.

Terminology—the notations used to designate complexes imply neither stoichiometries nor ligand distribution but merely the substrates of the complex based on the reaction mix and conditions used in the complex formation. RESULTS

Oligomeric GroEL Can Be Proteolysed by Trypsin in a Ligand-dependent Fashion—Fig. 1A represents a denaturing gel showing the time course for trypsin digestion of a GroEL-ADP-Mg complex. The parent band, representing a GroEL monomer, is digested, and a doublet appears consisting of bands with apparent molecular masses of ~31 and ~32 kDa. The intensities of the doublet bands increase over the time course and are approximately equal to each other at every time point, suggesting that they appeared together rather than one giving rise to the other upon further degradation. Other extremely faint bands appear but were not quantified since they do not represent a significant percentage of the digested GroEL monomer. The N-terminal sequences of the two bands of the doublet were determined, and they represent complementary sequences in a GroEL monomer; the upper band begins at glycine 269, and the lower band begins at the N terminus of the mature protein (alanine). Trypsin cleavage at Arg-268, in the apical domain, would produce two fragments of these approximate molecular masses and with the observed N-terminal sequences.

Fig. 1B is a semi-log plot representing the percentage of parent band (monomeric GroEL) remaining at various times during the trypsin digestion of the GroEL-ADP-Mg complex. The curve was best fit by a single exponential with the following parameters: \( k = 0.186 \) s\(^{-1}\), \( A_1 = 0.423 \), and \( A_2 = 0.516 \), where \( A_1 \) represents the fraction of undigested protein remaining at long proteolysis times. The results indicate that within the first 15 min of proteolysis approximately half of the monomers in the tetradecamer were cleaved at Arg-268, while the other half of the monomer population remained intact. Longer periods of digestion (up to 60 min) did not produce significantly more than 50% digestion (data not shown).

Fig. 2A shows a similar plot for the proteolysis of GroEL under different conditions. The upper curve (solid circles) represents trypsin proteolysis of the GroEL oligomer in the presence of 20 mM Mg\(^{2+}\). Very little digestion occurred (10% at most) without production of a doublet such as that seen in Fig. 1A. The lower curve (open squares) demonstrates the significant digestion of the unliganded GroEL oligomer. These data (open squares) were best fit by a single exponential with the following parameters: \( k = 0.067 \) s\(^{-1}\), \( A_1 = 0.852 \), and \( A_2 = 0.094 \). The digestion of unliganded GroEL also produced the doublet shown in Fig. 1A, but the percentage of the parent band...
To investigate the differences in proteolysis between unliganded and magnesium-ligated GroEL an experiment was conducted where the degree of proteolysis was measured at fixed times (15 or 30 min) at increasing concentrations of MgCl₂. The results, graphed in Fig. 2B, show that an increasing percentage of the monomers were protected from trypsin proteolysis as the MgCl₂ concentration was increased from 0 to 20 mM, with maximum protection afforded by 10–12 mM. This protection from trypsin proteolysis at increasing MgCl₂ concentrations is strikingly similar to the effect of increasing Mg²⁺ concentrations on the cross-linking of heptameric rings in GroEL (31).

Trypsin proteolysis of GroEL in the presence of ATP-Mg or AMP-PNP-Mg produced results similar to those seen with ADP-Mg. Fig. 3 is a semi-log plot comparing the results with the three nucleotides, showing that all three produced an oligomeric structure that was sensitive to trypsin proteolysis. It is clear that nucleotide binding alone and not hydrolysis produced the conformational change necessary to expose the clip site in the apical domain. In each of the three cases the same digestion products appeared on denaturing gels, with the bands of the doublet described above representing the predominant species (data not shown).

Proteolyzed GroEL Retains Its Tetradecameric Structure—Fig. 4 shows a non-denaturing gel of samples digested with trypsin for various times in the presence of ADP-Mg (treated as in Fig. 1). In this gel system the band shown corresponds to tetradecameric GroEL, which runs as a sharp band near the top of the gel and can be easily distinguished from monomers (not present/seen) that run as a smeared band and migrate considerably further into the gel (45). The single band representing the GroEL tetradecamer is initially sharp but becomes increasingly fuzzy as the proteolysis progresses, and the band appears slightly but progressively higher in the gel. The staining intensity of the single band also increases by approximately 50% across the series. These results demonstrate that the proteolysis did not disrupt the oligomeric structure of the GroEL but certainly did produce a band with altered electrophoretic and staining properties, suggesting a heterogeneous population of structures.

There was a question of whether the non-denaturing gels were visualizing tetradecamers that had not fallen apart or tetradecamers that had reassembled during electrophoresis. This question was addressed by running samples treated in the same manner as above on a gel permeation column (TSK-4000), a system that resolves monomers from tetradecamers. The samples of ADP-Mg-ligated GroEL treated with trypsin for 30 min eluted in the same volumes as controls with native, unperturbed GroEL, or ADP-Mg-ligated oligomers that had not been proteolyzed, indicating that the tetradecamers remained intact after proteolysis (data not shown). Two-dimensional polyacrylamide gel electrophoresis was also performed. The bands from non-denaturing gels were excised, dissolved in SDS sample buffer, and electrophoresed on denaturing gels. The results were exactly the same as when samples were run directly on denaturing gels, as shown in Fig. 1A. These results...
confirmed that the bands visualized on the non-denaturing gels consisted of cut and uncut monomers, which remained associated as oligomers after proteolysis.

Formation of a Ternary Complex Protects GroEL from Trypsin Proteolysis—Trypsin proteolysis of GroEL(10.4 µM monomer) was used to probe various complexes of GroEL with GroES and/or unfolded rhodanese. Fig. 5 is a semi-log plot showing the time course of trypsin digestion of GroEL in complex with GroES and/or non-native rhodanese. The two binary complexes, GroEL-GroES(1:1 molar ratio) (filled circles) and GroEL-rhodanese(1:2.5) (open diamonds), formed in the presence of ADP-Mg showed approximately the same digestion of GroEL as with nucleotide alone. Since both rhodanese and GroES were digested by trypsin in these experiments, it is difficult to draw simple conclusions from the results, although similar results for a GroEL-GroES complex have been previously reported (46). In contrast, the ternary complex of GroES-GroEL-rhodanese(2:1:2), formed in the presence of ADP-Mg, significantly protected the chaperonin from trypsin proteolysis (asterisks). These results are very similar to the protection from proteolysis displayed when the chaperonin was liganded only by Mg²⁺. This suggests either that an additional conformational change of GroEL occurs upon binding both proteins at one end of the oligomer, such that the unliganded heptamer is protected, or that the proteins bind at opposite ends of the chaperonin, physically blocking the sites of proteolysis on both heptameric rings.

Cations Protect GroEL from Trypsin Proteolysis—Fig. 6 shows the digestion time course of GroEL in the presence of either KCl (filled circles) or spermidine (open squares). Many faint proteolytic fragments were present on Coomassie-stained gels, but the doublet seen in Fig. 1 was either a very minor or non-existent digestion product (data not shown). Since the control for these experiments is proteolysis of the unliganded GroEL tetradecamer, the cations apparently protected approximately half of the sites from proteolysis. Although similar to the observations with Mg²⁺-liganded GroEL, these results are distinct in that the divalent cation protected almost 100% of the proteolytic sites whereas the monovalent or trivalent cations protected only 50%. In each of the experiments, the presence of proteolytic activity was verified as described under "Materials and Methods" to be certain that the cations did not adversely affect the protease (data not shown).

**DISCUSSION**

An understanding of how various ligands induce conformational changes in GroEL and the specific sites which become exposed is crucial to deciphering the molecular mechanism of chaperonin activity. We were able to follow such conformational changes by using the differential trypsin proteolysis of GroEL under various conditions. Initially, an important distinction arises, which should be carefully considered. Mg²⁺ alone shifts the conformation of the chaperonin from a state that is 100% susceptible to proteolysis to one that is completely protected from trypsin. This is consistent with published studies showing that 50% of the protein is cross-linked as a heptamer at Mg²⁺ concentrations of 10 mM or higher (31). Apparently, conditions that cause association of the monomers in one heptameric ring (such that cross-linking is facilitated) result in an oligomeric structure that is fully protected from trypsin proteolysis. Considering the concentration range of this effect and the fact that Mg²⁺ concentrations in E. coli may vary between 20 and 40 mM (47), it is more reasonable that the magnesium-ligated state of GroEL is the form that should be thought of as the physiological state (control) rather than the unliganded form.

Previous reports have suggested that nucleotides bind asymmetrically to the tetradecamer occupying only seven sites, presumably of a single heptamer ring (29, 48). The results presented here (Fig. 1, A and B) support those earlier studies by showing that 50% of the monomers become proteolytically sens-
The local flexibility of the protein is not affected by radecamer (50, 51). Although nucleotide binding induces intermediate domains and quaternary structural rearrangements in GroEL (27, 28), the local flexibility of the protein is not affected by formation of a GroEL-ATP complex (50). This suggests that structural regulation in the chaperonin exists at two different levels: 1) exposure of the reactive sites in one heptameric ring by nucleotide binding and/or hydrolysis; and 2) structural rearrangements upon protein-protein interaction that stabilize regions of local flexibility.

The effects of ions on the GroEL structure also suggest this multilevel basis for structural regulation. Divalent cations (i.e., Mg$^{2+}$ and Mn$^{2+}$) stimulate the GroEL ATPase activity, stimulate rapid cross-linking of monomers within a heptameric ring by glutaraldehyde, and completely prevent tryptophan proteolysis (31, 32) (Fig. 2A). Monovalent and polyvalent cations increase the exposure of hydrophobic surfaces that bind bis-ANS and prevent tryptophan proteolysis of the chaperonin by only 50% (31, 33) (Fig. 6). With the exception of K$^+$, monovalent and polyvalent cations do not affect ATPase activity. Taken all together, divalent cations appear to mediate intermonomer and heptamer/heptamer interactions, whereas monovalent and polyvalent cations appear to affect the local structure of monomers in a fashion that may possibly be localized to the apical domain.

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