The C-terminal Sequence of the Chaperonin GroES Is Required for Oligomerization*

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J effrey W. Seale and Paul M. Horowitz‡

From the Department of Biochemistry,
University of Texas Health Sciences Center,
San Antonio, Texas 78240 7760

The Escherichia coli protein GroES is a co-chaperonin that is able to assist GroEL in the refolding of proteins. GroES is a heptamer of seven identical subunits. Recent work has focused on the structural aspects of GroES. We have investigated the role of the C-terminal portion of GroES in its oligomerization. Limited proteolysis of GroES by carboxypeptidase Y gives a product in which the C-terminal 7 amino acid residues have been removed. Sedimentation velocity analysis reveals that the truncated form of GroES is unable to reassemble. The results presented here implicate the C-terminal sequence in intermonomer actions within the GroES oligomer. In addition, this work provides experimental verification of predictions implied in the recent x-ray determination of the GroES structure (Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. M., and Deisenhofer, J. Nature, in press).

The chaperonins are proteins that are essential for cell growth under normal conditions (1). Among their various functions, chaperonins have been shown to assist in the folding of many proteins. Widely studied representatives of the chaperonin family are the GroES and GroEL proteins of Escherichia coli (2, 3). Recent models for chaperonin action have the larger of the two proteins, GroEL, binding partially folded protein intermediates (4). GroES participates in the cycle by modulating the ATPase activity of GroEL (5–7). In addition, the release of partially folded polypeptides has been proposed to be modulated by GroES (8–10), possibly through the interaction of a mobile loop on GroES with GroEL (11).

Structurally, GroES is a heptamer composed of seven identical 10-kDa subunits (6). The subunits are organized into a single ring structure. An x-ray crystal structure of the oligomer has been recently solved and shows the core of the protein to be organized as a topologically irregular β-barrel. A recent study has shown that there exists a dynamic equilibrium between monomeric and oligomeric GroES (12). In that report, GroES was shown to dissociate with a half time of a few minutes and a dissociation constant of $1 \times 10^{-38}$ M$^{-1}$. Those results indicate that, as with GroEL (13), monomeric GroES may play an important functional role in the mechanism of chaperonin action.

We have investigated the role of the C-terminal region of GroES in the oligomerization of the protein. The C-terminal sequence of GroES is resistant to proteolysis by carboxypeptidase Y in the absence of structural perturbants. However, in the presence of moderate concentrations of urea, a portion of the C-terminal sequence of GroES becomes susceptible to proteolysis. Under conditions that have been previously shown to allow for reassembly of urea denatured GroES, the truncated species is no longer able to oligomerize. Amino acid analysis reveals that the C-terminal 7 amino acid residues are released from GroES, giving the truncated species. Structurally, these residues appear to be important for the integrity of the monomer interface.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were of highest purity available. Carboxypeptidase Y was purchased from Sigma and used without further purification.

Protein Purification—GroES was purified from E. coli containing the pND5 plasmid bearing the groESL operon as described previously (14) with slight modification. GroES that had been purified according to this method was further purified by gel filtration on a Sephacryl S-200 column. Fractions containing purified GroES were pooled, concentrated, and stored in 70% ammonium sulfate at 4 °C.

Proteolysis of GroES with Carboxypeptidase Y—GroES (0.5 mg/ml) was incubated in 50 mM sodium phosphate, pH 6.2, and 100 mM NaCl containing various concentrations of urea (see figure legends) and 1% (w/v) carboxypeptidase Y. Digestion times were determined by removing aliquots at various times and analyzing the peptides by SDS-polyacrylamide gel electrophoresis (15) and native gel electrophoresis (16).

Reassembly of GroES—GroES (0.5 mg/ml) was digested with carboxypeptidase Y (2%, w/v) in the presence of 3 M urea for 90 min. The entire sample was then dialyzed overnight at 4 °C against 10 mM Tris-HCl, pH 7.8, and 100 mM NaCl to remove the urea. The oligomerization state of truncated GroES was determined by sedimentation velocity analysis using a Beckman XL-A analytical ultracentrifuge. For each run, GroES concentration was 0.5 mg/ml. The temperature was kept constant at 25 °C. The rotor speed was 60,000 rpm for all samples. The scans were analyzed by the method of van Holde and Weischet (17) using the UltraScan data collection and analysis program (B. Demeler, Department of Biochemistry, University of Texas Health Sciences Center, San Antonio, TX). All data were corrected for buffer density and viscosity.

Amino Acid Analysis of Truncated GroES—GroES (0.5 mg/ml) was treated with carboxypeptidase Y (2% w/v) in the presence of 3 M urea for 90 min. The sample was then placed in an Ultrafree-MC microconcentrator unit with a M, 5000 cut-off (Millipore) in order to separate the amino acids from the protein mixture. The samples were centrifuged at 4500 $\times g$ until approximately 85% of the sample had passed through the membrane. Next, 200 μl of distilled H$2O$ was added to the top portion of the filter unit. The sample was again centrifuged at 4500 $\times g$ until approximately 90% of the sample had passed through the membrane. The entire filtrate, approximately 500 μl, was saved for amino acid analysis.

Amino acid analysis was performed using a Beckman 7300 analyzer using the ninhydrin reaction after the amino acids had been separated on a 20-cm high resolution ion exchange column. Detection of the derivatized amino acids was at 570 and 440 nm. The retention time and peak heights of the samples were compared with known standards to determine amino acid identity and quantity. The results from the amino acid analysis were compared to the published sequence of GroES to determine the site of truncation (18).

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‡To whom correspondence should be addressed.

1 Hunt, J. F., Weaver, A. J., Landry, S. L., Gierasch, L., and Deisenhofer, J. Nature, in press.
RESULTS AND DISCUSSION

Limited Proteolysis of GroES by Carboxypeptidase Y Removes the C-terminal 7 Amino Acids—Fig. 1A shows an SDS gel demonstrating the proteolysis of GroES by carboxypeptidase Y in the presence of moderate concentrations of urea. GroES in the absence of urea is not susceptible to proteolysis by carboxypeptidase Y (Fig. 1A, lane 0 M). In the presence of either 3 M urea (Fig. 1A, lane 3 M) or 4 M urea (Fig. 1A, lane 4 M), a single proteolytic product is formed after 90 min. This product appears to be stable since subsequent digestion products do not appear on this time scale (data not shown). Examination of the properties of carboxypeptidase Y (19) along with the primary structure of GroES does not reveal any indication that proteolysis should be inherently blocked at any point within 20 residues in the C-terminal sequence of GroES. In fact, after 24 h, significant further proteolysis is observed (data not shown). These results suggest that the initial truncation is retarded by the conformation of GroES.

The proteolytic product also demonstrates an altered mobility during native gel electrophoresis (Fig. 1B, lanes 3 M and 4 M). Conditions were chosen from previous studies that would allow for reassembly of oligomers by dilution into an equal volume of buffer followed by electrophoresis (lanes marked by B). Likewise, dilution of the sample into an equal volume of 9 M urea (lanes marked U) would allow for the dissociation of oligomeric GroES. A comparison in Fig. 1B of lane ES with lane U at 0 M urea shows that electrophoresis allows for the reassembly of samples that were originally at concentrations as high as 4.5 M urea. The digestion product has an altered mobility irrespective of the dilution conditions prior to electrophoresis.

Analysis of the amino acids released from the limited proteolysis of GroES give the following ratios of amino acids: A:I:E = 2:2:1:1:1:1. When matched with the published sequence of GroES (18), this sequence corresponds to the 7 C-terminal amino acids of GroES (highlighted in bold): ... EVLIMS E SDILAI VEA.

This sequence corresponds to the predicted C-terminal strand in the GroES structure (20, 21). One of these reports implicates this C-terminal strand in intermonomer interactions (21).

Truncated GroES Is Unable to Oligomerize—Recent reports have described the reversible oligomerization of GroES under native conditions (12) or from a urea-denatured state. The oligomerization state of the truncated GroES was investigated by sedimentation velocity analysis. GroES that had been digested with carboxypeptidase Y in the presence of 3 M urea was dialyzed against buffer to remove the urea and allow for reassembly of GroES. These dialysis conditions are sufficient for the reassembly of urea-dissociated GroES.2 Fig. 2 shows the sedimentation velocity behavior of truncated GroES. The majority of the species has an $s_{20w}$ value of approximately 1 S. This value corresponds to the value previously reported for monomeric GroES.2 The sample also contains some larger species, as indicated by the nonuniformity of the plot (17). Native gel electrophoresis reveals a small amount of heptameric GroES remaining in this sample prior to ultracentrifugation (data not shown) that would account for the heterogeneity of the plot. It is important to note that under conditions where the proteolysis is stopped prior to complete formation of the truncated species, the products consist of only heptameric and monomeric GroES. There are no other oligomeric species formed, i.e. dimers, trimers, etc. (data not shown).

Several recent reports have focused on the structural characteristic of GroES1 (20, 21). It is believed that the oligomeric structure of GroES serves to coordinate the hydrolysis of ATP (22–24). It has been reported recently that GroES dissociates at relatively low concentrations (12). The dissociation of GroES suggests that the monomeric species may play a role in the chaperonin mechanism.

The present work emphasizes the importance of the C-terminal sequence of GroES in the oligomerization of the protein. When these results are analyzed in the context of the recently solved x-ray crystal structure,1 the reasons become clear. The C-terminal 7 residues form the last $\beta$-strand. This $\beta$-strand makes the primary contacts with the N terminus of a neighboring monomer forming the interface between monomers. This interface is rich in flexible side chains, and the interface is described as having structural plasticity. In summary, the production of monomeric GroES by limited proteolysis provides a new avenue for the study of GroES function.

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