Nucleotides Reveal Polynucleotide Phosphorylase Activity from Conventionally Purified GroEL*

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GroEL, as conventionally purified, can be incubated with nucleotides to produce high molecular weight material with the observation that GroEL is essential for E. coli. This material is most clearly demonstrated when samples are subjected to gel filtration under conditions where GroEL is monomeric. There is a time-dependent increase in the high molecular weight material that occurs on incubation with ADP or, more slowly, with ATP. This material is generated during incubation, and none is present in the initial samples. Experiments with nucleases, proteases, radiolabeled nucleotides, and chemical cleavage reagents demonstrate that the high molecular weight material is polyadenylic acid whose formation is inhibited by phosphate. These results are consistent with the GroEL samples containing polynucleotide phosphorylase activity. Nondenaturing gels stained with acridine orange, after incubation in ADP, reveal that the activity producing the poly(A) coelectrophoreses with authentic polynucleotide phosphorylase. Conditions that remove the tryptophan-like fluorescence from preparations of GroEL also remove the PNPase activity. Thus, this activity is not associated with GroEL itself. The results are consistent with reports that GroEL can associate with RNase E and with other studies showing that RNase E and PNPase can form complexes. Thus, the present experiments support suggestions that GroEL can participate in multiprotein complexes that are involved in mRNA processing and degradation.

The Escherichia coli chaperonin GroEL has been studied mostly for its ability to assist protein folding by binding and stabilizing non-native folding intermediates. This activity, together with the observation that GroEL is essential for E. coli, has been the focus of suggestions as to the role of this protein in the cell. However, the generality of the requirement for GroEL in the folding of most proteins has been called into question, since it has been calculated that there is only enough GroEL to refold ~5% of the proteins in E. coli (1). At the same time, several investigators have proposed additional roles for this chaperonin including involvement in protein turnover (2) and RNA processing and degradation (3, 4). In these cases, it has been suggested that GroEL forms part of an assembly with other active proteins to provide the overall function(s).

Degradation of mRNA appears to require the coordination and control of exo- and endonuclease activities. A protein complex has been identified that can mediate mRNA degradation in E. coli since it contains a number of polypeptides including the exoribonuclease, polynucleotide phosphorylase (PNPase), and the endoribonuclease, RNase E, in addition to an activity that impedes PNPase activity at stem loop structures. It was suggested that this organization could be important for mRNA processing (5, 6). Complexes that were prepared by procedures involving high ionic strength (5) or high ionic strength and detergents (2) did not contain GroEL. Other studies have indicated that GroEL can co-purify and functionally interact with RNase E activity (3), and it has been recently demonstrated that a protein complex containing GroEL can be isolated that binds and protects mRNA (4). These latter studies did not report the presence of PNPase activity.

GroEL, as commonly isolated, copurifies with other polypeptide chains. These have most often been ascribed to the diverse set of polypeptides that interact with GroEL as it exerts its folding function. Recent methods have been developed to produce GroEL that appears quite homogeneous by gel staining, although the preparations often contain distinct tryptophan-like fluorescence, which is unexpected since GroEL contains no tryptophan in its sequence. Very recently it has been possible to treat GroEL in a way that removes this tryptophan fluorescence while retaining a fully functional chaperonin with respect to protein folding (7). Since GroEL is large (M_r = 840,000), and it binds modestly sized proteins with a small stoichiometry (1:1), it is difficult to detect single proteins bound to GroEL oligomers. For example, 10 μg of GroEL would contain 0.4 μg of rhodanese (M_r = 33,000) at full occupancy, so that it might be difficult to detect bound rhodanese in partially occupied GroEL after its isolation.

These considerations raise the possibility that GroEL may be a constituent of complexes in which advantage can be taken of its ability to provide extensive binding surfaces that could serve to organize activities and modify or sequester bound polypeptides or nucleotides. Therefore, it is of interest to determine whether distinct activities can be detected in association with GroEL. In this work, we report that polynucleotide phosphorylase activity can be found in association with highly purified GroEL, and that the presence of this protein can contribute to the commonly observed tryptophan-like fluorescence of these preparations. The partial occupancy after purification and the poor detectability by commonly applied procedures has complicated the detection in the usual preparations. The sensitivity of GroEL interactions to the solution conditions used in different isolation procedures can contribute to the variability of finding PNPase in each case. The present work also has a practical caveat, in that detection of radiolabeled nucleotide coeluting with GroEL does not necessarily imply protein bind-

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The abbreviations used are: PNPase, polynucleotide phosphorylase; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
ing since GroEL and polynucleotide products coelute in some of the commonly used chromatographic systems.

**EXPERIMENTAL PROCEDURES**

**Materials**

Proteinase K (catalog number PE558) and polyribonucleotide-orthophosphate nucleotidytransferase (PNPase; polyribonucleotide phosphatase, EC 2.7.7.8, catalog number PE624) were from Sigma. DNase I from bovine pancreas was from Boehringer Mannheim. Ribonuclease A (DNase-free) was prepared from Sigma RNase type IA and kindly supplied by Dr. John Lee (University of Texas Health Science Center, San Antonio, TX). All other reagents were analytical grade.

**Purification of GroEL**

Conventional Purification---The chaperonin GroEL was purified from lysates of E. coli cells bearing the multicopy plasmid pGroESL (8). This conventional preparation was a modified version of published protocols (9, 10) as described previously (8, 7). The protein from this preparation was used for most of the work reported here.

Affi-Gel Blue Treatment---Conventionally purified GroEL was treated with Affi-Gel Blue (Bio-Rad) as described previously (11, 12) to remove some 280 absorbing impurities. Briefly, 160 mg of GroEL were loaded onto an Affi-Gel Blue column (1.5 × 16.5 cm) that had been equilibrated with 50 mM Tris-HCl, pH 7.5, that contained 50 mM KCl. After a brief period, the column was eluted with the equilibration buffer. GroEL eluted while the impurities were retained on the column. As reported previously (11), GroEL prepared by this treatment contained a considerably reduced but still significant tryptophan-like fluorescence.

Assembly-Disassembly Preparation---GroEL was prepared as described previously by a method (Method I of Ref. 7) that involves disassembly of the GroEL 14-subunit oligomer and subsequent reassembly of the monomers (7). This procedure produces GroEL that contains very few contaminating polypeptides and shows little detectable tryptophan-like fluorescence. The GroEL so prepared is fully functional in its ability to assist the refolding of the enzyme rhodanese (7).

**Gel Permeation Chromatography---HPLC gel permeation chromatography, and the present studies were performed to determine the nature and origin of the material that is under a. The main question was whether the material was released from GroEL by the treatment or whether it was being formed during incubation. If it was being formed, was it by GroEL itself or from increasing times. There is a time-dependent increase in material absorbing at the 230 nm wavelength used for detection that elutes in the void volume (peak a, Fig. 1). This peak is well separated from the monomeric GroEL (peak b, Fig. 1). The curves represent chromatography of 300-μg samples injected at different times after dilution from 8 mM urea (1-h incubation) into 50 mM triethanolamine acetate, pH 7.5, containing 5 mM MgADP to give a final urea concentration of 0.8 M. Incubations were at room temperature. Under these conditions, the GroEL remains largely monomeric. The monomers elute at b. The peak at a increases with time, and the profiles shown at correspond, from the bottom, to: 0, 1, 2, and 3 h after dilution, respectively. GroEL 14-mer would elute between a and b and corresponds to the small shoulder.

The material collected under peak a had a UV maximum at 260 nm characteristic of nucleotides. The appearance of peak a required nucleotide and, although it could be observed on incubation with ATP, the peak appeared more slowly than when the incubation was with ADP. In addition, the appearance of peak a with ATP was associated with hydrolysis. Thus, an experiment was performed in which GroEL (400 μl at 1 mg/ml) was incubated with 58 × 10^6 cpm of γ-labeled ATP, and 120 μl of the mixture was chromatographed on the TSK column. No counts were recovered under peak a for incubations of 0, 2, or 4 h. All counts were in the included volume. When the same experiment was repeated with α-labeled ATP using 36 × 10^6 cpm, 112,000 cpm were recovered under peak a.

These results demonstrated that nucleotide-like material was contained in peak a and raised the question of its origin. It is important to realize that these gel filtration experiments would not clearly separate 14-mers from the material under peak a, and, thus, previous experiments of this type that detected some nucleotide under the GroEL peak may not have been monitoring nucleotide bound to the GroEL. In fact, a previous report indicated that some preparations of GroEL could generate high molecular weight nucleotide-like material (17). Further experiments were done here to determine the cause of these anomalies. Fig. 1 shows HPLC gel permeation chromatography of conventionally purified GroEL run on a TSK 4000 column after denaturation in 8 mM urea and subsequent renaturation with MgADP for increasing times. The curves represent chromatography of 300-μg samples injected at different times after dilution from 8 mM urea (1-h incubation) into 50 mM triethanolamine acetate, pH 7.5, containing 5 mM MgADP to give a final urea concentration of 0.8 M. Incubations were at room temperature. Under these conditions, the GroEL remains largely monomeric. The monomers elute at b. The peak at a increases with time, and the profiles shown at correspond, from the bottom, to: 0, 1, 2, and 3 h after dilution, respectively. GroEL 14-mer corresponds to the small shoulder between a and b.

**Fluorescence and UV Spectra---Fluorescence spectra were determined with an SLM 500C fluorometer (SLM Instruments, Urbana, IL). UV spectra were determined with a Milton Roy Spectronic 3000 diode array spectrophotometer (Houston, TX).**

**RESULTS AND DISCUSSION**

High molecular weight material forms upon incubation of conventionally purified GroEL in ADP. A recently described protocol for purification of GroEL involves disassembly of the 14-mer in the presence of ADP (7). The monomeric GroEL showed anomalous behavior when subjected to gel permeation chromatography, and the present studies were performed to document and determine the cause of these anomalies. Fig. 1 shows HPLC gel permeation chromatography of conventionally purified GroEL run on a TSK 4000 column after denaturation in 8 mM urea and subsequent renaturation with MgADP for increasing times. There is a time-dependent increase in material absorbing at the 230 nm wavelength used for detection that elutes in the void volume (peak a, Fig. 1). This peak is well separated from the monomeric GroEL (peak b, Fig. 1). The curves represent chromatography of 300-μg samples injected at different times after dilution from 8 mM urea (1-h incubation) into 50 mM triethanolamine acetate, pH 7.5, containing 5 mM MgADP to give a final urea concentration of 0.8 M. Incubations were at room temperature. Under these conditions, the GroEL remains largely monomeric. The monomers elute at b. The peak at a increases with time, and the profiles shown at correspond, from the bottom, to: 0, 1, 2, and 3 h after dilution, respectively. GroEL 14-mer corresponds to the small shoulder between a and b.
some copurified activity residing on a distinct protein?

Fig. 2 shows nondenaturing gels of samples from the gel filtration fractions in Fig. 1 run under conditions that can resolve GroEL monomers and 14-mers. At \( t = 0 \) there are monomers and some 14-mers, but there is no material in the lanes corresponding to fractions from peak \( a \) (first four fractions at \( t = 0 \)). The gel on the right shows that after 5 h there is material under peak \( a \) that stains with silver, but barely enters the gel. These latter bands do not stain with Coomassie Brilliant Blue. This can be seen more clearly in Fig. 3 which shows some of these fractions run on SDS gels and stained with silver (left) or Coomassie (right). The material from the GroEL monomer peak stains with both Coomassie and silver, while the material under peak \( a \) stains only with silver (labeled 260). This reflects either the amount or nature of the material under peak \( a \). Nucleotides stain with silver, but stain poorly with Coomassie.

Advantage was taken of the fact that the material from peak \( a \) in Fig. 1 could be silver-stained to test its sensitivity to several treatments. No band could be observed after treating for 30 min a sample of this material containing 0.35 \( A_{260} \) (from a stock solution containing 35.2 \( A_{260} \)) with 0.5 \( M \) KOH or 0.1 mg/ml RNase. The band could still be observed after treatment with DNase (0.1 mg/ml) or protease K (0.1 mg/ml). Thus, the material in peak \( a \) has the characteristics of an RNA-type polymer.

In separate experiments, fractions from gel chromatography of GroEL not treated with ADP were subjected to electrophoresis. Fig. 4 shows denaturing (upper) and native (lower) gels for samples taken from the peak fractions of a sample of conventionally purified GroEL chromatographed on Sephacryl S400 without treatment with ADP. The SDS gel shows that fractions across the peak appear homogeneous by Coomassie staining. In addition, the lanes denoted by \( A \) contain samples of conventionally purified GroEL that were treated with Affi-Gel Blue as described under “Experimental Procedures” in a procedure that reduces the level of tryptophan fluorescence associated with the GroEL. The lanes marked \( P \) contain samples of \( E. coli \) PNPase (Sigma). The lower, nondenaturing gels show samples from the same fractions as above that were electrophoresed in the absence of any preincubation with ADP. If these gels were directly stained with Coomassie, only the upper bands would be disclosed (data not shown). Instead, the gels were incubated overnight in MgADP at 4°C before being treated with acridine orange to detect polynucleotide products. Only the lower bands were observed as reddish bands on an orange background. These bands coelectrophoresed with the acridine orange bands that were observed with authentic PNPase. This postelectrophoretic staining detected proteins in the lower bands by virtue of their ability to produce polynucleotides in the gel. Samples of GroEL that were treated with Affi-Gel Blue showed substantially reduced quantities of the acridine orange band (lane \( A \), gel 3). After staining with acridine orange, the gels were counterstained with Coomassie Brilliant Blue which after this total protocol showed both the upper and lower bands as demonstrated in Fig. 4 (gels 3 and 4). These results indicate that
proteins that remained associated with GroEL throughout the purification procedure could be separated by this electrophoretic protocol.

Fluorescence spectra were used to test whether proteins could be observed under peak a. Fluorescence spectra were measured with excitation at 280 or 295 nm for a sample of pooled fractions from peak a after TSK 4000 chromatography. These spectra were characteristic of tryptophan-containing proteins, and, in each case, the wavelength maximum was at 346 nm.

Samples of GroEL that were prepared by the assembly-disassembly protocol were subjected to the same treatments as described above. No evidence of a polynucleotide product was observed under any conditions, and the fluorescence spectrum was that of a protein containing only tyrosine as previously reported (7).

Those peptides that have been found associated with GroEL after purification have always been considered to be contaminating peptides that reflect the binding of a large and diverse group of polypeptides in various states of synthesis and folding. The only reported activity that has been associated with purified GroEL is RNase E. The results presented here demonstrate that polynucleotide phosphorylase activity can be detected in samples of highly purified GroEL that has been prepared under conditions that should be capable of separating these proteins. The results suggest that there are conditions that can result in the association of these proteins. These types of experiments cannot determine whether this association is an in vivo state of these proteins as opposed to a fortuitous interaction consequent to their adventitious admixture during preparation. It is interesting, though, that separate studies have demonstrated on the one hand that RNase E and PNPase are associated, while on the other hand RNase E and GroEL can be associated. It is tempting to speculate that these three proteins can be associated at some stage of their in vivo existence. This would support the role of GroEL as a participant in the processing and turnover of mRNA (3).

The polynucleotide phosphorylase is difficult to detect in the presence of GroEL. It stains poorly with common protein stains, and it would be a minor component if present with the much larger GroEL. Conventionally purified GroEL is commonly reported to contain the equivalent of 0.2 mol of tryptophan/monomer based on fluorescence. Based on the molecular weight of 77,122 and the content of 2 Trp per PNPase polypeptide, the observed Trp content would correspond to a complex containing 13% PNPase by weight.

The polynucleotide product can be formed by ATP as well as ADP, although more slowly. It is expected that PNPase activity is specific for ADP. Presumably, the slow ATPase activity of GroEL can form ADP which then can be polymerized by the PNPase.

On a practical note, the finding of PNPase activity with commonly available GroEL represents a caveat for assessing the binding of nucleotides by gel permeation methods that separate the large GroEL from the smaller mononucleotides. On many commonly used columns, the polynucleotide products will coelute with the GroEL oligomer, thus leading to the inference that the nucleotide has become incorporated into the GroEL.

Overall, GroEL may be involved in a wide range of functions that can take advantage of its ability to bind partially folded polypeptides and perhaps polynucleotides and folded proteins with appropriate structures such as surface loops or interactive surfaces.

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