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Archives of Biochemistry and Biophysics 434 (2005) 352–357
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Asymmetric binding of membrane proteins to GroEL

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Received 13 October 2004, and in revised form 15 November 2004
Available online 8 December 2004

Abstract

The interaction of GroEL with non-native soluble proteins has been studied intensively and structure–function relationships have been established in considerable detail. Recently, we found that GroEL is also able to bind membrane proteins in the absence of detergents and deliver them to liposomes in a biologically active state. Here, we report that three well-studied membrane proteins (bacteriorhodopsin, LacY, and the bacteriophage λ holin) bind asymmetrically to tetradecameric GroEL. Each of the membrane proteins was visualized in one of the center cavities of GroEL using single particle analysis.

Keywords: Membrane proteins; GroEL; 3D Structure; Solubilization; Electron microscopy; Single particle analysis; 3D Reconstruction

The tetradecameric protein GroEL and its eukaryotic homolog hsp60 have been the subject of intensive study, in terms of its role in folding non-native polypeptides as well as misfolded proteins. GroEL encompasses two chambers of about 85,000 Å^3 and employs an ATP-driven multi-step cycle for folding during which each chamber is alternately filled and emptied, and the co-chaperonin GroES is bound and released [cf. 1]. GroEL, in concert with its co-chaperonin GroES, is considered to be a major component of the cellular machinery for refolding misfolded cytosolic proteins [cf. 1]. Several reports have shown that GroEL can form complexes with native membrane proteins in vitro, in the absence of detergent [2–4]. The complexes are formed efficiently provided that GroEL is present during removal of detergent by dialysis and the amount of membrane protein per tetradecamer does not exceed a mass approximately equal to the putative binding capacity of a single chamber, which is thought to be approximately 60 kDa [5,6]. Experiments with the well-studied multitopic membrane protein bacteriorhodopsin (BR),[1] the light-driven proton pump from *Halobacterium halobium* with 7α-helical transmembrane domains (TMDs), showed that BR retains native conformation upon binding to GroEL and has a limit of two molecules per chaperonin. The binding was sensitive to ATP, suggesting that the domain movements that accompany ATP-binding lead directly to rapid ejection of the substrate protein [2]. In addition, both BR and the bacteriophage λ holin S105, a 12-kDa

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[1] *Abbreviations used:* BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BR, bacteriorhodopsin; DDM, dodecyl-β-D-maltoside; EBB, empigen BB; EM, electron microscopy; FSC, Fourier shell correlation; LacY, lactose permease; S105, bacteriophage λ holin; TMD, transmembrane domain.

0003-9861/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2004.11.021

protein with three putative TMDs responsible for forming holes in the host membranes at an exact time point during the end of the lytic cycle, were shown to be efficiently delivered from these complexes to liposomes. Moreover, both proteins were shown to retain biological function upon insertion [2,7]. These findings raise the possibility that GroEL may play a role in the insertion of membrane proteins into the lipid bilayer in vivo. To more fully characterize this potentially important new role for the Hsp60 class of chaperones, the results of single particle analysis of GroEL complexed with three different membrane proteins, i.e., BR, S105, and 12 TMD lactose symporter LacY, are presented.

Materials and methods

Protein purification and complex formation

GroEL, BR, and the λ holin, S105, were purified as previously described [7]. To form complexes between S105 or BR and GroEL, 800 μl of 1% EBB, 20 mM BES, and 0.5 M NaCl, pH 7.6, was placed in a tube, after which 100 μl of a GroEL solution and 100 μl of membrane protein in the same buffer were added. At each step, the solution was mixed by pipette. For most experiments, the final concentration of GroEL was 100 μg/ml and the concentration of membrane protein was adjusted to achieve the desired molar ratio to the tetradecameric chaperonin. The 1 ml solution containing detergent-solubilized membrane protein and GroEL was placed into a dialysis bag, and dialyzed against 500 ml of 20 mM BES, 0.5 M NaCl, pH 7.6, supplemented with Calbiosorb Bio-Beads, according to the manufacturer's instructions. Buffer and beads were changed every 8 h. Dialysis was continued until there was quantitative precipitation in a control sample containing the subject protein but with GroEL replaced by an equal mass of bovine serum albumin (Calbiochem). In these experiments, the efficacy of detergent removal was assessed using calcein-loaded liposomes as previously described [8]. In all cases, detergent was reduced to less than 10% of its critical micellar concentration.

LacY was purified as described previously [9]. For complex formation between LacY and GroEL, LacY in 20 mM Tris (50 mM KCl, 0.3% DDM, pH 7.4) and GroEL in 50 mM Tris (50 mM KCl, 1 mM DTT, pH 7.4) were combined at a 1:1 stoichiometric ratio. The buffer of the reaction mixture contained 50 mM Tris (pH 7.4), 50 mM KCl, and 0.008% DDM, and the protein concentrations were 2.5 μM for both, LacY and GroEL. The reaction mixture (200 μl) was then injected into a dialysis cassette with a 10 kDa MWCO (Slide-A-Lyzer, Pierce) and dialyzed for 2 h at room temperature against 2 × 500 ml dialysis buffer (50 mM Tris, 50 mM KCl, pH 7.4) in the presence of 50 g Bio-Beads. The complete removal of DDM under the conditions employed was verified using [^{14}C]DDM. The dialyzate was loaded onto a sucrose density gradient (in steps from 9, 10–18% w/v; 1.2 ml total volume) and centrifuged for 2 h 20 min at 4 °C using an RCF_{avg} of 200,000*g*. One hundred twenty microliter fractions were collected and analyzed for protein composition by SDS–PAGE. Fractions containing LacY and GroEL were combined and used for electron microscopic analysis. With regards to GroEL, all molar concentrations and stoichiometries mentioned refer to the tetradecamer.

Electron microscopy

For electron microscopy, GroEL, GroEL + BR, and GroEL + S105 holin complexes were dialyzed into 25 mM Tris (pH 7.5, 10 mM KCl, 10 mM $MgCl_2$). GroEL + LacY complexes remained in the same buffer as described above. Specimens were prepared using a modification of the standard droplet technique as described by Harris and Agutter [10]. Three microliters of GroEL (0.2 mg protein/ml) or GroEL-membrane protein complexes was directly applied to a freshly glow-discharged carbon-coated copper grid (G400), washed with distilled water, and negatively stained with an aqueous solution of uranyl acetate (1% w/v, pH 4.25). Images were recorded at a calibrated magnification of 40,800× in a JEOL 1200EX transmission electron microscope operated at an acceleration voltage of 100 kV.

Image analysis

Selected micrographs were digitized using a Leafscan 45 microdensitometer with 2.57 Å/pixel at the specimen level. Single particle analysis was carried out with the EMAN software package [11]. For each data set, a minimum of 1000 particles were used for the reconstructions. Selection of particles was carried out using the semi-automatic BOXER routine within EMAN. After preprocessing (filtering, centering, etc.), a first 3D model was calculated with 10% averaged top and side views. The first structure was iteratively refined until no changes in the 3D reconstruction were observed as judged by Fourier shell correlation (FSC). Rendering thresholds were set to account for the overall protein mass at the GroEL:membrane protein stoichiometric ratios used. 3D reconstructions were visualized using the Vis5d program (http://vis5d.sourceforge.net; courtesy of S.G. Johnson, J.Edwards, and W. Hibbard). Although tetradecameric GroEL has an inherent D7 symmetry (one axis of 7-fold rotational symmetry orthogonally intersected by an additional axis of 2-fold rotational symmetry), the lower C7 (one 7-fold axis only) symmetry operations were employed for all data sets to avoid forcing a distribution of densities over both cavities. The good contrast observed with the raw particles as well as

the high symmetry of GroEL facilitated the alignment process and obviated the need for processing larger data sets. Docking of X-ray crystallographic structures to electron microscopic data were performed using the VMD software package [12].

Results and discussion

Single-molecule 3D reconstructions of GroEL

To assess the capability of single-molecule analysis to detect GroEL cargo, 3D reconstructions of negatively stained GroEL tetradecamers were calculated (Fig. 1) and compared to the X-ray crystal structure of GroEL (1OEL) [13] blurred to ~1.5 nm resolution using a Gaussian filter. There is very good agreement between the two structures, plainly revealing a separation of the monomeric subunits of GroEL as well as their differentiation into apical and equatorial domains. It should also be noted that even details such as the protein deficits at the heptamer interface, which are beyond the nominal confidence limit of ~2.5 nm as per the 0.5 FSC criterion, can be readily discerned (Figs. 2A and B, arrows). At this resolution, no mass was detected in the chambers of the reconstructions of GroEL. Inspection of the angular distribution in the asymmetric triangle indicated that 3D Fourier space was appropriately sampled (Fig. 1). Representative raw data, class averages, and reprojections from the 3D analysis are shown in Fig. 1, together with an assessment of the reliability of the final 3D reconstructions by FSC [14].

Reconstructions of GroEL-membrane protein complexes

Previously, we have shown that complexes can be formed between BR molecules (seven TMDs; molecular mass ~30 kDa) and GroEL; titration experiments demonstrated that, under the dialysis conditions used, two molecules of BR can bind per GroEL tetradecamer [2]. To determine the mode of BR binding, i.e., (i) one BR in each chamber, (ii) both in one chamber, or (iii) a mixture of these modes, we performed single particle analysis of negatively stained BR–GroEL complexes formed under saturating conditions. Central sections taken through the 3D volume revealed small but significant and reproducible additional density located in the center of one cavity. The densities are non-contiguous with the densities that delineate the cavity surface (Fig. 2). As a control, 3D reconstructions of unloaded GroEL were also rendered as if additional mass was present, but no additional densities were observed inside the cavities.

Similar results were obtained with complexes formed between GroEL and the λ holin, S105, which has three TMDs and a mass of about 12 kDa. These complexes were formed at a ratio of six S105 molecules per GroEL

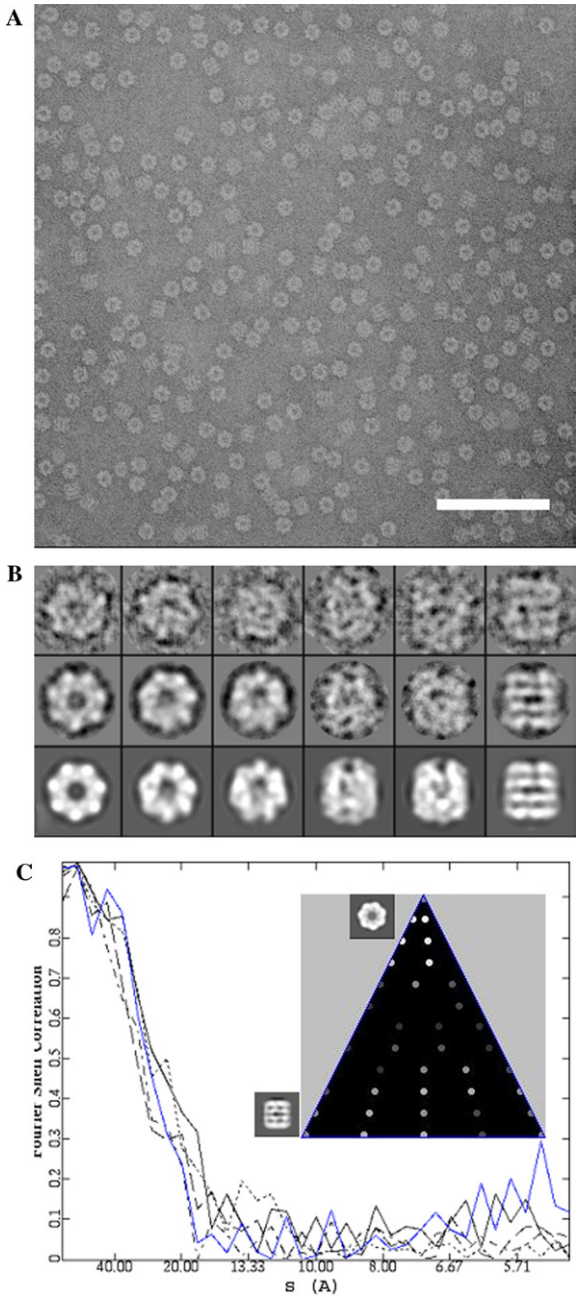

Fig. 1. Raw data, image processing, and resolution assessment. (A) Representative micrograph of negatively stained GroEL single particles (scale bar is 100 nm). (B) Gallery of raw particles (top), class averages (middle), and reprojections of GroEL (bottom) with top views, intermediate projections, and side-on view (box sizes are 20 × 20 nm). (C) Fourier shell correlation (FSC) of X-ray and EM structures of GroEL, indicating a resolution of approximately 2.5 nm. The inset shows the asymmetric triangle and the angular distribution of typical class averages obtained.

[7]. Again, small but reproducible extra densities were observed centrally located in one chamber (Fig. 2), which is consistent with previous results obtained using nanogold-labelled S105 [7].

Lactose permease (LacY; 12 TMDs; 45.6 kDa) is one of the few integral membrane proteins for which a

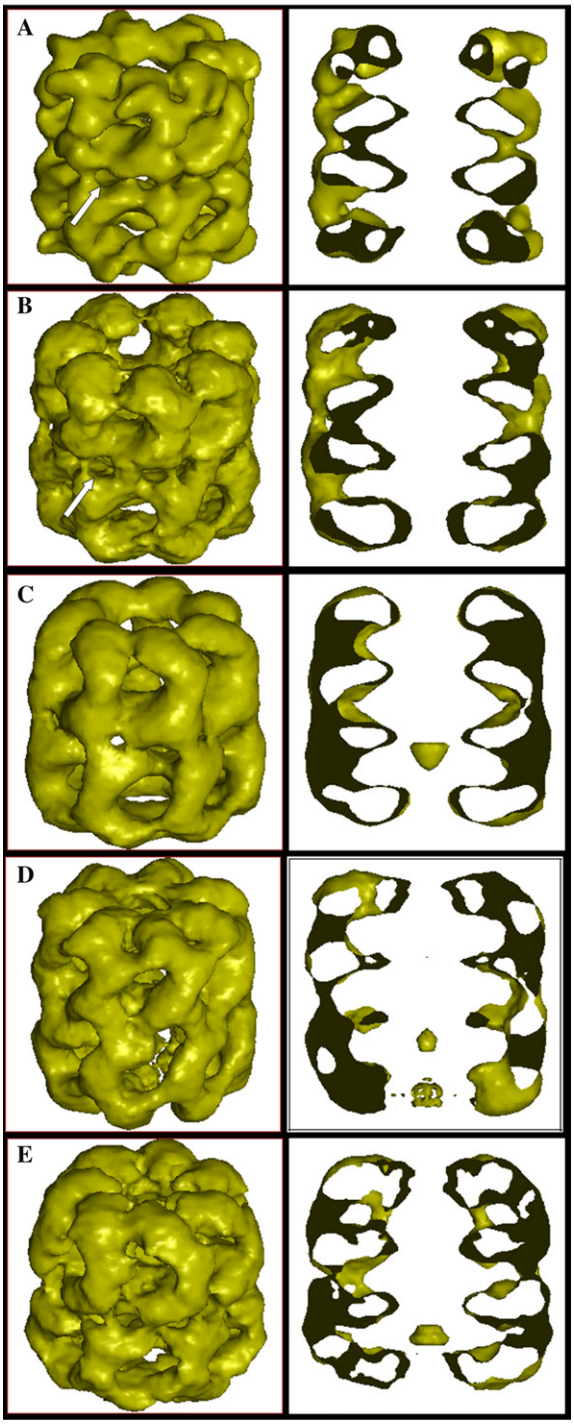

Fig. 2. 3D reconstructions of GroEL and GroEL–membrane protein complexes. (A) The crystal structure of GroEL (1OEL) filtered down to 1.5 nm resolution. (B) Reconstruction of empty GroEL. (C) GroEL + LacY. (D) GroEL + BR. (E) GroEL + S105. The surface rendering threshold was set to correspond to a molecular mass of 840 kDa. The left-hand column shows slightly tilted side-on views of GroEL and the right-hand column shows center sections parallel to the 7-fold axis.

high-resolution X-ray crystal structure is available and thus represents an advantageous system for characterizing the GroEL-membrane protein complexes. However, LacY is not stably solubilized in detergent, and even at 1:1 LacY:GroEL, the best yield of GroEL–LacY complexes formed after dialysis was ~0.5 LacY per GroEL, with the remainder forming insoluble precipitates [2]. The 3D reconstructions of images of these complexes also revealed extra density in the center of one chamber of GroEL (Fig. 2).

Overall, in the 3D reconstructions, some GroEL-specific densities undergo slight changes when comparing GroEL alone with the GroEL:LacY, GroEL:BR, and GroEL:S105 complexes. These may reflect the domain rearrangements that are expected to take place upon interaction with the cargo proteins, if the binding of native membrane proteins follows a pattern similar to that of non-native soluble proteins. In the recent crystal structure of symmetric GroEL bound to fourteen 12 amino-acid peptides [15], small domain rotations were reported, but these movements are too subtle to be readily visualized by electron microscopy. However, the conformational changes in the chaperone induced by the binding events reported here may be significantly larger because the protein loads correspond to much larger masses. Moreover, the cargo is native protein primarily composed of highly hydrophobic transmembrane domains. Therefore, the conformational stability of these proteins may require a much more pronounced domain movement within the apical domain and the inner chamber to be accommodated and protected from the aqueous environment. However, conclusions as to GroEL-specific conformational changes upon membrane protein binding must await higher resolution studies.

Modeling the binding of membrane proteins to GroEL

The crystal structures of BR [16] and LacY [17] allowed us to test whether a reasonable structure could be modeled in which two molecules of BR or one molecule of LacY occupy one cavity of tetradecameric GroEL. Docking was performed by translating the crystal structures of the cargo proteins into the cavity of the GroEL-membrane protein reconstruction. Positioning and orientation was performed by superimposing the crystal structures with the extra densities observed while avoiding any overlap with GroEL-specific densities (Fig. 3). Even though no attempt was made to minimize the binding enthalpy of these structures, the models indicate that the binding observed in vitro can be rationalized in terms of the known tertiary structures. It should be noted that by these criteria, two BR monomers can be loaded either parallel to or orthogonal to the long axis of the GroEL central cavity. However, the likelihood of the orthogonal orientation is deemed very low considering the hydrophobic nature of the 14 TMDs in the two BR molecules and the lumenal surface of the apical domain. To this end, Fig. 3 only depicts the more likely, parallel arrangement.

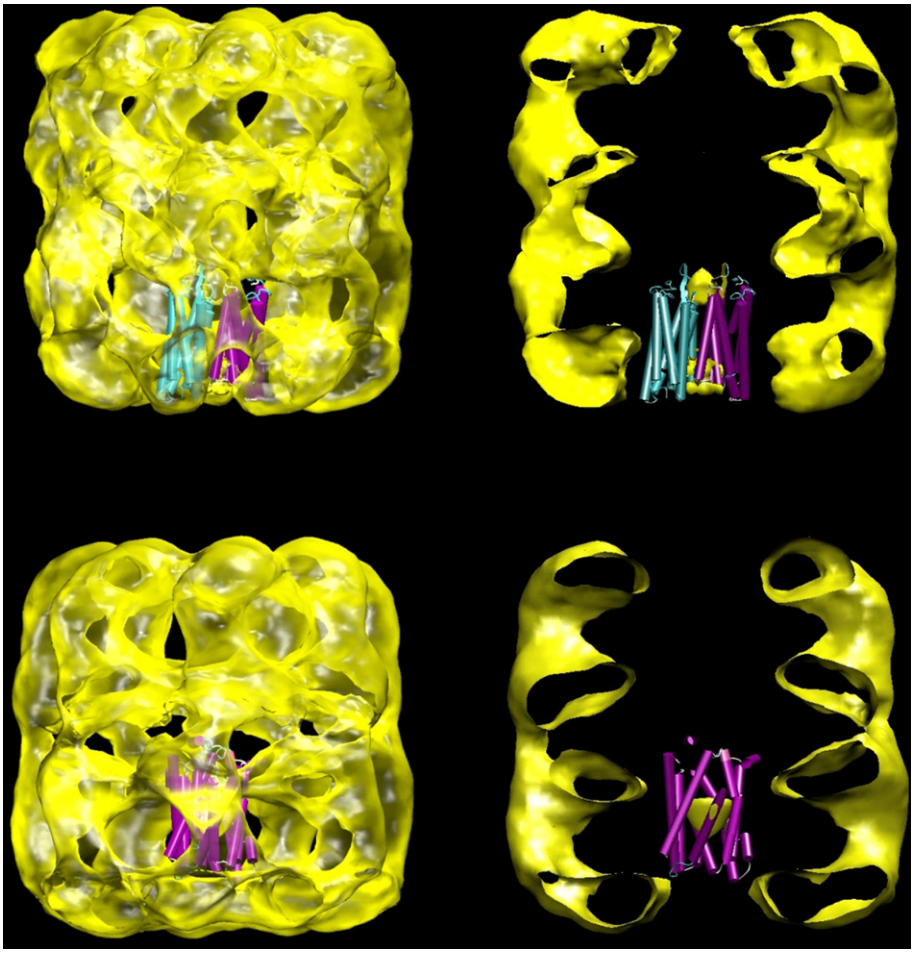

Fig. 3. Representations of GroEL–membrane protein reconstructions docked to atomic resolution crystal structures. In the right column the GroEL maps have been clipped to allow observation of internal features whilst in the left column the EM densities were rendered semi-transparent to allow visualization of the docked crystal structures. Note that due to the clipping of densities, not all helices are visible in the crystal structures. Top row: GroEL–BR map docked to the BR crystal structure 1QM8 with the BR dimer 2-fold parallel to the GroEL 7-fold. Bottom row: LacY crystal monomer (1PV7) docked to the EM GroEL–LacY map to coincide with the LacY-specific density observed in the reconstruction.

The models illustrate the approximate amount of extra mass which should be visible in the complexes. The maximum "payload" as per cavity of GroEL in the absence of GroES has been estimated to equate to approximately 60 kDa [5,6]. However, it remains still to be determined whether the same rules apply when comparing GroEL-dependent folding of soluble proteins with GroEL's ability to solubilize membrane proteins. The size limit of the cavities of GroEL have only been tested with unfolded soluble proteins, which are larger than the condensed fold of membrane proteins. Hence, it is likely that the cavity of GroEL could could house a larger mass of membrane protein than unfolded soluble protein.

According to our previous results using titrations of the membrane protein cargo and to the 3D reconstructions reported here, the mass of the cargo accommodated inside a single cavity would be 46–72 kDa [i.e., one LacY molecule (45.6 kDa), two BR molecules (2×26 kDa), or six molecules of S105 (6×12 kDa)]. A volume corresponding to such a mass would be expected to be larger than the extra densities observed (Fig. 2). However, it is not known whether negative stain can penetrate the chamber efficiently in the presence of cargo protein. Moreover, the positioning of the cargo protein relative to GroEL will not necessarily follow the 7-fold rotational symmetry, causing randomly oriented molecules to be averaged out during the processing, except for those densities at the point of continuous occupancy. These limitations have been also observed in single-molecule analyses of complexes formed between GroEL and soluble proteins [18] and, in any case, do not detract from the essential point that with each of the membrane proteins examined, the extra densities were always restricted to one cavity. This means that with the binding of membrane proteins, a negative cooperativity similar to the one reported for non-native soluble proteins is observed [19]. If this were not the case, one would expect a distribution of the cargo protein-specific densities over both cavities. It is also important to note that these extra

densities along the longitudinal axis of GroEL extend far beyond the apical regions. In the case of LacY, the density is distal from the cavity opening and proximal to the equatorial domains. Although this density represents only a part of the LacY molecule, it is possible that some interactions are also occurring between the soluble loops of LacY and the intermediate-equatorial domains of GroEL. Given the loading of LacY into GroEL is less efficient than with BR or S105, the finding of comparable extra densities in the LacY–GroEL complexes compared to the other two cases may indicate that the permease has less conformational freedom within the chamber.

Finally, the three complexes described herein share common themes: (i) in each case, only one of the chambers is loaded, (ii) the loading is independent of the GroES co-chaperonin, and (iii) the addition of ATP causes unloading of the chambers ([2], M. Svrakic and H.R. Kaback, unpublished data). The uniform behavior of these integral membrane proteins in the GroEL system suggests that there may be an in vivo role for GroEL in membrane integration. Of particular interest is the holin S105, which, although it functions in the membrane, has been shown to be sec-independent (E. Ramanculov, R. Young, unpublished data). Preliminary immunoprecipitation experiments using anti-GroEL antibodies with cytosolic extracts of induced lysogens suggest that S105 forms complexes with GroEL in vivo (J.F. Deaton and R. Young, unpublished results). If this in vivo role can be confirmed, then it will be of interest to determine the orientation of the proteins bound in the GroEL chamber and assess what aspects of GroEL structure are required for the interaction with the bilayer.

With the LacY, BR, and GroEL crystal structures known, it could be possible to probe the GroEL-membrane protein interaction to higher resolution using cryo-electron microscopy on complexes for which there is a symmetry match. Moreover, it may be possible to obtain crystal structures of membrane proteins by co-crystallizing them with GroEL in the absence of detergent.

Acknowledgments

The authors thank Jim Sacchettini and his group for continuing helpful and collegial input and criticism of this work. This work was supported by PHS Grant GM27077 (R.F.Y.), a Robert A. Welch Foundation grant (R.F.Y.) and the Life Sciences Task Force (R.F.Y. and A.H.).

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