SOLUBILIZATION AND FUNCTIONAL

ANALYSIS OF THE LAMBDA HOLIN

A Dissertation

by

JOHN FRANKLIN DEATON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Biochemistry

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Approved as to the style and content by:

Ryland F. Young (Chair of Committee)	David O. Peterson (Member)
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ABSTRACT

Solubilization and Functional Analysis of the Lambda Holin.

(August 2004)

John Franklin Deaton, B.S., Berry College

Chair of Advisory Committee: Dr. Ryland F. Young

The 105aa lambda S protein is the prototype holin, S accumulates in the cytoplasmic membrane during late gene expression until, at a time programmed into its primary structure, it disrupts the membrane and allows the lambda lysozyme, R, to attack the cell wall. In this study, a zwitterionic detergent Empigen BB, was used to extract and purify the lambda holin S. In Empigen BB, CD analysis on S gave 54% alpha helical content, consistent with 3 TM domains, which has been reported by other *in vivo* studies. Empigen BB-purified S can be exchanged into a chaotropic solution by dialysis and reconstituted into preformed lipid vesicles for activity assays. When diluted to fluorescein-loaded suspensions of liposomes, different chaotrope-solubilized S alleles caused dye release reflective of their *in vivo* phenotypes. The problem was the low efficiency of delivery of S to the liposomes. Unfortunately, dye loaded liposomes are highly sensitive to any detergent, making it necessary to find other ways to solubilize S.

GroEL, a chaperonin from *E. coli*, is responsible for folding and refolding globular proteins *in vitro*. It has also been reported that GroEL improves the ability of a membrane protein synthesized *in vitro* to insert post-translationally into liposomes. This work will investigate the behavior of GroEL towards membrane proteins. The first of two membrane

proteins studied in this respect is Bacteriorhodopsin (BR), a membrane proton pump, from *H. halibium.* The second is the105aa S protein, a prototype holin from bacteriophage lambda. Holin and BR subjected to detergent removal in the presence of GroEL remained in solution, while in the control sample (without GroEL) S and BR precipitated. "GroEL-solubilized" holin still retained its lesion forming activity and solubilized BR maintained its proton pumping ability, detected by using a liposome dye activity assay unique to each protein. This approach may be applicable to other systems requiring detergent- or chaotrope-free preparation of membrane proteins. Finally, these results suggest that GroEL may be involved in the insertion of integral membrane proteins into the lipid bilayer, a role heretofore unsuspected.

DEDICATION

This dissertation is dedicated to Pamela, Brandon and Cody, for without their physical and psychological support, I would never have succeeded. My children make the long days and hard work bearable.

ACKNOWLEDGEMENTS

I am grateful to have the love and support of my grandparents, aunt and mother on my quest for higher education. I owe one particular individual, Sue Stiles, for financial support that enabled me to attend graduate school.

I acknowledge my boss and friend, Ry Young. His patience, understanding, encouragement and insight guided me through my work. I am indebted to Doug Struck also for his guidance and patience during my time in the lab. Doug is an integral part of the lab. I can not put into words how thankful I am for my committee members; James Sacchettini, David Peterson, and Andreas Holzenburg, time, comments and help. I also thank Norma Teetes for her invaluable time, organization and listening. I would also like to acknowledge the great friendships I acquired working with everyone over the years, in particular, Tom Bernhardt, Erlan Ramanculov, Brenley McIntosh, Christos Savva, Rebecca White, Min Xu, Anh Tran, Carrie Langlais, Joel Berry, Taehyun Park, Liz Summer and Maria King. I would like to thank Ron Kaback and Maya Svracic for communication of unpublished results, helpful discussions, and LacY protein. Thanks also go to Mark Krebs and Hagan Bayley for invaluable advice about bacteriorhodopsin purification. I am indebted to the following for gifts of purified protein: Amy Davidson for MalGFK2; Arjan Bormans and Michael Manson for Tar; James Bowie for DAGK.

I am grateful to Prasad Reddy for his generous gift of a groEL over-expression strain. Work reported here was supported by PHS grant GM27099 to Ryland Young and by funding for the Program for Membrane Structure and Function from the Life Science Task Force at Texas A&M University to Ryland Young and Andreas Holzenburg. Andreas

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ABBREVIATIONS

BR = bacteriorhodopsin

BES = N,N'-bis(2-hydroxyethyl)-2-2aminoethanesulfonic acid

DAGK = diacylglycerol kinase

BSA = bovine serum albumin

DM = n-dodecyl a-D-maltoside

DMSO = dimethylsulfoxide

OG = n-octyl-b-D-glucopyranoside

INV = inverted membrane vesicles

TMD = transmembrane domain

EBB = Empigen BB

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS = Tris-buffered saline

CHAPTER I

AN INTRODUCTION TO *E.coli* GroEL, LAMBDA HOLIN

AND *H. salinarium* BACTERIORHODOPSIN

INTRODUCTION

Membrane proteins

Membrane proteins participate in some of the most important and interesting cellular processes including energy transduction, cell signaling, membrane excitability, secretion and immune recognition. However, the molecular mechanisms by which membrane proteins accomplish these tasks are largely unknown. Approximately 30 to 40 percent of the proteins encoded by prokaryotic and eukaryotic genomes are membrane proteins, and 70 percent or more of current pharmaceuticals appear to act on membrane proteins. For these reasons, correlating the structure of membrane proteins with their function represents one of the most important challenges for biochemical analysis and structural biology. Unfortunately, due to their one defining physical characteristic, namely their water insolubility, only a few structures of membrane proteins have been determined by X-ray crystallography or through reconstruction from images obtained by electron microscopy.

With the exception of proteins found in the outer membrane of Gram-negative bacteria, the transmembrane domains (TMD) of membrane proteins are uniformly α-helical in structure in order to satisfy main chain hydrogen-bonding requirements. TMDs are

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This dissertation follows the style of *Molecular Microbiology*.

typically 16-24 residues in length, and the preponderance of these are hydrophobic in nature allowing thermodynamically favorable interactions with the hydrocarbon core of the membrane in which they reside or with the hydrophobic surfaces of other TMDs.

The purification and characterization of membrane proteins are significantly more difficult than for soluble proteins due to the presence of one or more hydrophobic TMDs. Detergent screens are required to determine optimal conditions not only for extraction of the membrane protein from the lipid bilayer but also for its continued solubility and function once it is extracted. Even if a detergent can be found that meets these requirements, a final obstacle still must be overcome to fully characterize that protein *in vitro*, namely its reconstitution into a defined lipid bilayer. The most common method of reconstitution is to solubilize both the membrane protein and lipid bilayer in detergent, mix them together and then remove the detergent by dialysis (Rigaud and Levy, 2003). Although this technique has met with some success, it suffers from two major limitations. First, proteins reconstituted in this fashion are likely to adopt a random orientation with respect to the lipid bilayer. This would make it difficult, if not impossible, to study the function of a protein that catalyzed or facilitated a process that showed directionality with respect to the membrane. Second, the efficiency of reconstitution by detergent dialysis is highly variable (Rigaud and Levy, 2003) so that a large fraction of the input protein can be lost through aggregation and precipitation. Clearly, there is a need for methods that allow the directional insertion of membrane proteins into preformed bilayers with a reproducible and high efficiency.

Bochkareva reported the first successful post-translational solubilization and membrane insertion of a multispanning membrane protein, lactose permease, by the *E.coli* chaperonin GroEL (Bochkareva *et al.*, 1996). In a membrane free extract, biosynthesis of [^{35}S] Met labeled LacY leads to complete aggregation. In contrast, the addition of GroEL to the reaction mixture results in around 30% of the LacY synthesized being soluble and in a form that co-sediments with GroEL in sucrose gradients. Furthermore, incubating these presumptive GroEL-LacY complexes with Mg^{2+}, ATP and *E.coli* inverted inner membrane vesicles (INV) at 37C, allowed detectable amounts of LacY to be delivered to the INV. Proteolytic digestion and ligand binding assays indicated that the post-translationally inserted LacY molecules adopted a functional conformation.

Although largely ignored, this initial report suggests that membrane proteins can complex with GroEL and be delivered from these complexes to membranes. This raises the possibility that GroEL might be generally useful for the *in vitro* reconstitution of membrane proteins. Moreover, the post-translational insertion of nascent proteins into the cytoplasmic membrane, a process thought to be widespread in eubacteria, may require the participation of chaperonins such as GroEL *in vivo*.

My interest in the work of Bochkareva (Bochkareva *et al.*, 1996) stems from that the fact the objective of my thesis research is to establish a reconstituted system for functional studies of the S protein of bacteriophage lambda. The S105 protein accumulates in the cytoplasmic membrane of infected hosts and, at a time specified by its amino acid sequence, oligomerizes in the membrane to form lesions which we refer to as holes; S105 belongs to a class of proteins known as holins (Young and Blasi,1995).

Although the stimulus to trigger the oligomerization of S105 is not known, premature triggering can be brought about by the collapse of the electrochemical proton gradient that normally exists across the membrane. Thus, there is a good reason to believe that a membrane potential is necessary to maintain S105 in its pre-hole state. To recapitulate S105 function *in vitro*, it will be a necessary to find a means to insert S105 protein into a preformed membrane across which an electrochemical proton gradient has been established. Additionally, there must be a mechanism to collapse and re-establish this gradient. The system I have decided to explore uses lipid vesicles containing the light-driven proton pump, bacteriorhodopsin, as the target for S105 insertion. It is envisioned that GroEL's ability to facilitate the post-translational insertion of proteins into membranes will be exploited for the reproducible formation of the target vesicles and the delivery of S105 to them.

GroEL

GroEL was first discovered by C.P. Georgopoulos by selecting mutants of *E.coli* that specifically block assembly of bacteriophage lambda heads (Georgopoulos and Eisen, 1974). These mutants were called *groE*, because all *groE* mutants can be compensated by mutation of the phage gene E (the major structural subunit of the phage head). Polar insertions in the *groEL* operon showed that groEL was essential for cell viability. When *GroEL* was depleted by a temperature sensitive *groEL* mutant grown at non-permissive temperatures (Horwich *et al.*, 1993), changes in the levels of many proteins were noted, but no particular protein was identified as both GroEL-dependent and essential for cell survival. A better method of depleting GroEL was to replace the native *groE* chromosomal promoter

region with the *araC* gene and *araBAD* promoter, which allowed for study of proteins that required GroEL for cell viability in the absence of heat shock (McLennan and Masters, 1998). Using this system, The protein *dapA*, dihydropicolinate synthase, was shown to require GroEL. However, *dapA* was not the only essential gene, since exogenous *dapA* delays, but does not inhibit cell death when GroEL was depleted (McLennan and Masters, 1998)).

Pulse-chase labeling followed by isolation of GroEL-polypeptide complexes by immuno-precipitation showed that a diverse set of proteins between 10-55kDa interact with GroEL, accounting for 10%-15% of all cytoplasmic proteins under normal growth conditions. Furthermore, in the presence of heat stress, 30% or more of all cytoplasmic proteins interact with GroEL (Ewalt *et al.*, 1997).

The interaction of GroEL with lipid membranes was studied by simulation of one side of the bilayer with injection of GroEL into the aqueous phase beneath a phospholipid monolayer. The consequent change in surface pressure was measured while keeping the surface area constant (Torok *et al.*, 1997). It was found that binding of GroEL to the monolayer is affected by its composition and physical state, and requires the presence of the last 16 amino acids of the C-terminal tail of GroEL Not only was GroEL able to bind lipid monolayers, but the ATPase and protein folding activity of GroEL was retained, indicating GroEL may participate in the folding or stabilizing of denatured membrane proteins during heat shock.

GroEL is a molecular chaperone that recognizes and selectively binds nonnative proteins, but not native proteins, to form relatively stable complexes (Walter and Buchner,

2002). GroEL facilitates folding of bound non-native proteins in combination with its cochaperonin GroES and ATP. It not only prevents aggregation but also simultaneously allows partially folded intermediates to fold in an environment conductive to stabilizing the native state (Ellis, 2003). The crystal and electron microscopic structures of GroEL have been determined. GroEL consists of 14 identical subunits in two stacked heptameric rings, each containing a central cavity. Each cavity consist of three domains which are, from top to bottom; the apical domain, forming the mouth of the central cavity (Fig.1- 1), the intermediate domain, forming a hinge; and the equatorial domain that binds ATP/ADP. All three domains make the cavity of GroEL. The GroEL cavity is ~45 angstrom in diameter, with a cavity volume of 175,000 angstrom cubed (Kusmierczyk and Martin, 2001) and can hold roughly a 60KDa protein. The binding of ATP causes the hydrophobic cavity lining, which the unfolded substrate binds before GroES binding, to become much more polar while substantially increasing the size of the cavity (Ellis, 2003). The ability of GroEL to bind to, stabilize and help fold misfolded proteins has been studied extensively.

According to current models for GroEL-assisted protein folding, the unfolded protein, with exposed hydrophobic surfaces first binds the apical domain (active cavity), causing a slight conformational change in GroEL. The new conformation has increased the affinity for ATP and GroES. Upon binding of ATP and GroES (Fenton and Horwich, 2003), GroEL undergoes a large conformational change, exposing hydrophilic residues in

the cavity while burying the previously exposed hydrophobic residues. Exposing the bound, unfolded protein to this new environment is thought to stimulate it folding. Hydrolysis of ATP weakens the binding of GroES bound to the active cavity and increases binding of GroES and ATP to the inactive cavity. GroES and ATP binding to the inactive cavity releases GroES and unfolded/folded protein from the active cavity. Release of ADP from the active GroES/ATP cavity resets GroEL for another round of folding (Fig. 1-2).

Several GroEL mutants have been important in identifying target proteins and which cochaperonins are required to fold the target protein. D87K GroEL binds but does not release non-native proteins, providing a method to "trap" non native proteins with a fast on/off rate (Fenton *et al.*, 1994). E238A GroEL fails to bind GroES, which can be used to determine if target proteins require GroES binding to GroEL in order to fold. Finally, Δ532-548 GroEL fails to bind membranes *in vitro*, which can be used as a control to determine if GroEL can interact with membrane proteins that are imbedded in the membrane.
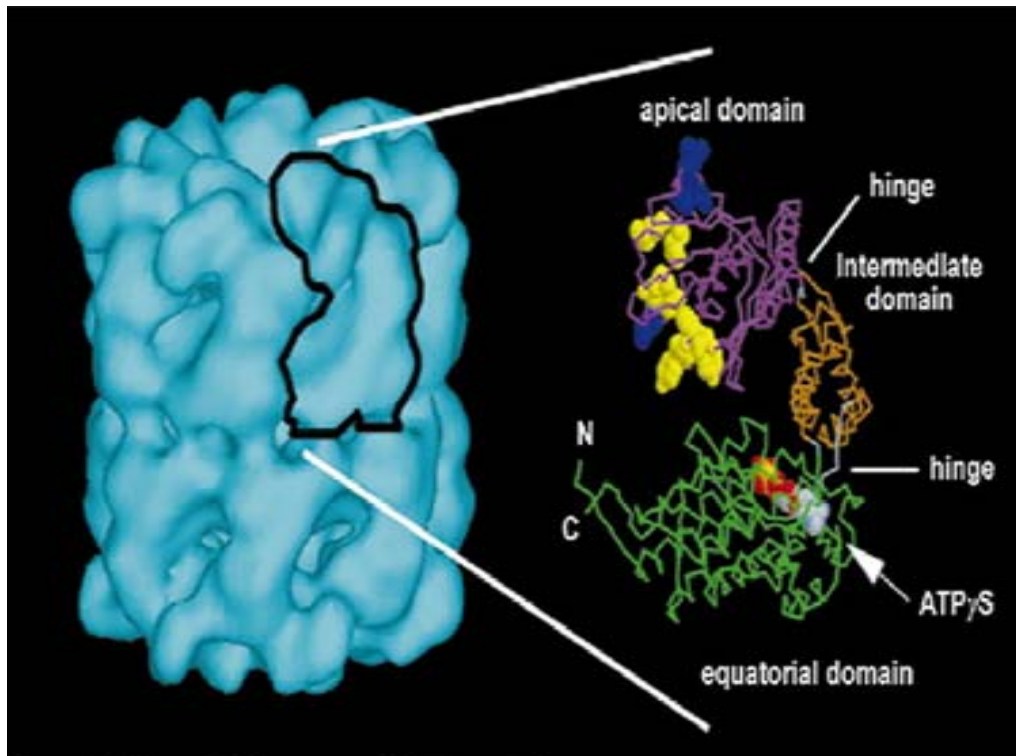
Figure 1-1. Crystal structure of GroEL and diagram of its domains.

The equatorial domain which forms the interface between the two 7-mer chambers and binds nucleotides. The intermediate domain which connects the equatorial and apical domains. The apical domain which binds hydrophobic polypeptides and GroES (Rye and Chen, et al. 1996).
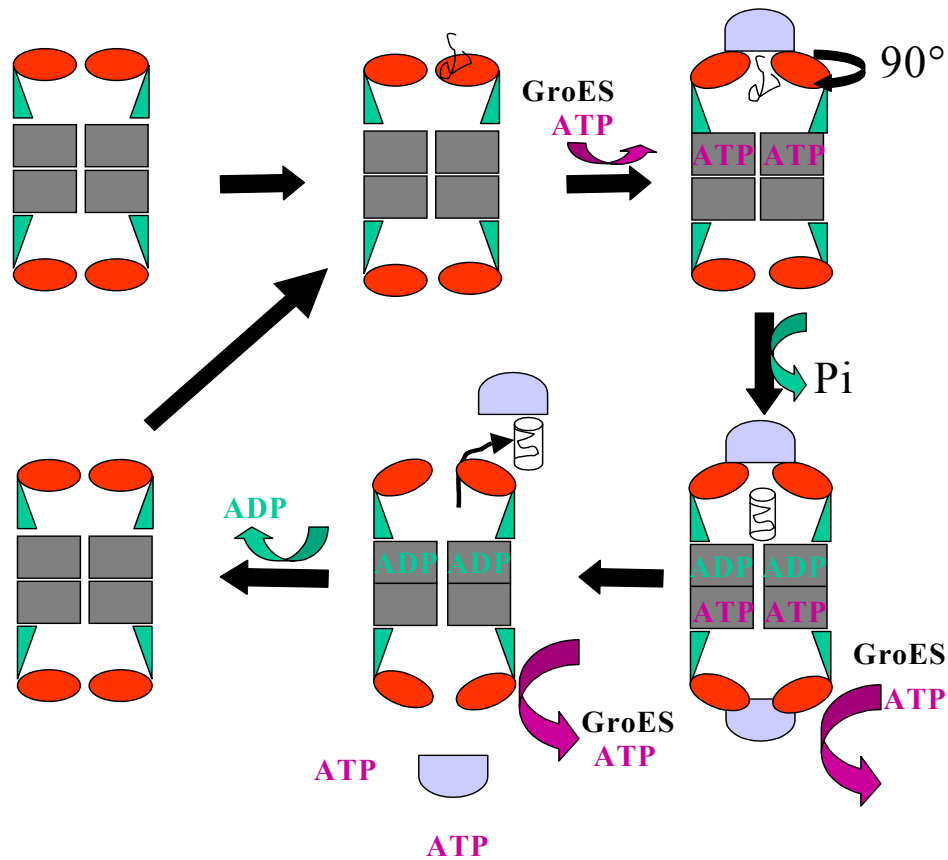
Figure 1-2. Cartoon model of the GroEL folding pathway.

Non-native polypeptide binds the apical domain of GroEL. Upon binding, minor conformational changes in the apical domain of GroEL increases the affinity for GroES and ATP. GroES and ATP bind to the apical and equatorial domains respectively, forces the non native polypeptide further into the cavity and triggering a major conformational change. The apical domain rotates 60 degrees up and 90 degrees around, exposing the hydrophilic residues and burying the hydrophobic residues. While the protein is folding in this hydrophilic environment, ATP is hydrolyzed and weakens the binding of GroES to the apical domain and increasing the affinity of GroES and ATP to the inactive GroEL chamber. Upon binding of GroES and ATP to the inactive chamber, GroES and the folded proteins are released from the active chamber. Release of ATP, GroES from the inactive chamber and ADP from the active chamber, resets GroEL for another round of activity.

Lambda Holin

The holins are easily the most diverse group of functional homologs among membrane proteins. They are small proteins involved in host lysis by all double-stranded DNA bacteriophages. For most phages, the role of the holin is to form a permeabilizing lesion in the cytoplasmic membrane, allowing the endolysin, a phage-encoded soluble muralytic enzyme, to gain access to its substrate, the cell wall (Fig.1-3). Nothing is known about the structure of the lesion, or "hole", except that it consists of oligomers of the holin. The hole, in the case of the lambda holin, is non-specific and capable of allowing release of pre-folded proteins up to at least 400 kDa (Wang, Deaton, and Young, 2003). Besides the formation of the hole, the other essential role of the holin is the timing of the lytic event. Holins are benign while accumulating in the membrane during the vegetative cycle but spontaneously trigger to form the lethal holes at a precisely-scheduled time; because of this essential timing function, holins can be considered the simplest biological clocks. More than 130 holins have been identified by genetics and genomics; the majority have 3 TMD (class 1 holin which include S) or 2 TMD (class II which include S^{21} of phage 21). Many holin genes also encode an antiholin, by virtue of dual translational starts like *S*, but in other cases, the antiholin is encoded by a separate gene.

The lambda lysis cassette consist of 4 genes, S (holin), R (transglycosylase), Rz (unknown function) and Rz1 (unknown function) (Fig. 1-4). Only S and R are absolutely required for host cell lysis under standard laboratory conditions. Rz and Rz1 are required

A.
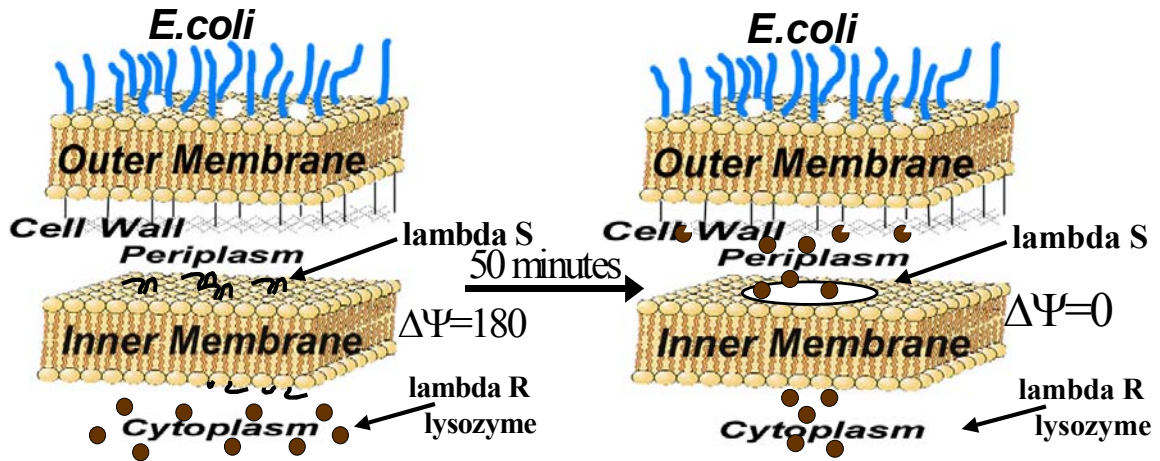
B.
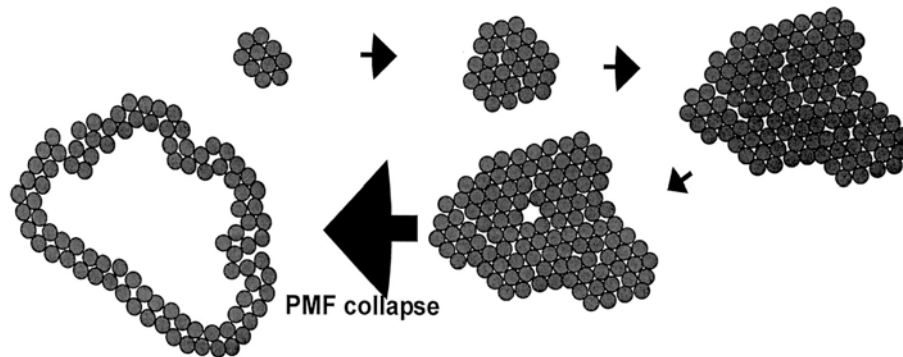
Figure 1-3. Models for holin activity and accumulation.

A. Model of lambda lysis. S (cytoplasmic membrane) and R (cytoplasm) accumulate during late gene expression, at a given time (50 minutes after induction under standard laboratory conditions), the holin triggers. The triggering results in several events; a lesion is formed in the inner membrane, the membrane potential collapses and the endolysin is released into the periplasm where it degrades the cell wall. These events result in lysis of the cell due to osmotic shock. **B**. A model of how S oligomerizes during accumulation in the inner membrane until triggering occurs.

A.
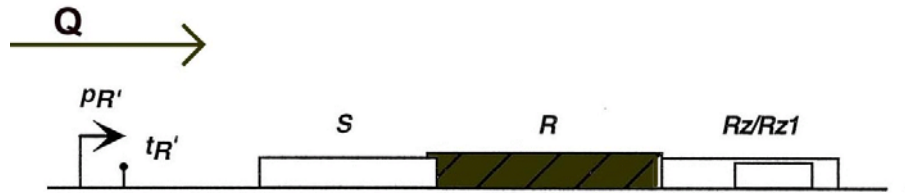
B.
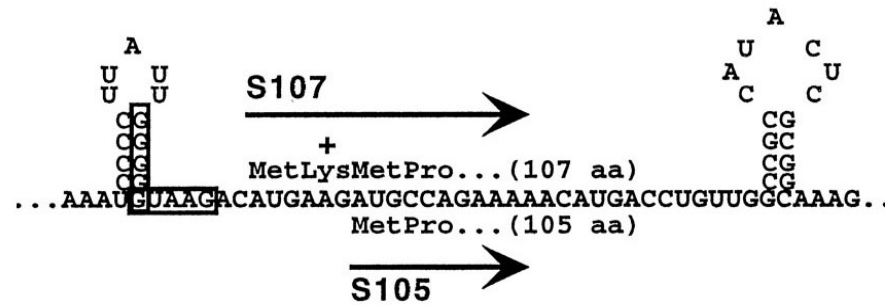
Figure 1-4. Transcriptional and translation control of the S gene.

A. The lambda overlapping lysis cassette, which is controlled by the lambda antiterminator Q. **B**. The dual start motif of the lambda holin. Expression is regulated by the RNA stem loops flanking the holin initiation site.

for lysis only when the outer membrane is stabilized by millimolar concentrations of divalent cations. By using toeprinting (primer extension), it was shown that the two S start codons are served by two adjacent Shine-Dalgarno sequences (Blasi, Nam, et al. 1989). It has been shown using immunological and biochemical methods that the two predicted protein products, S105 and S107, are detectable *in vivo* as stable, membrane-bound molecules (Grundling, Smith, et al. 2000)(Fig. 1-3). Furthermore, S107 acts as an inhibitor in trans, and that its inhibitory function is entirely defined by the positively charged Lys2 residue (Blasi, Chang, et al. 1990). Under standard laboratory conditions S105 lyses *E.coli* at around 45 minutes alone and delayed until 50 minutes in the presence of S107 , at 2 to 1 holin to anti-holin. S107 may just "trap" S105 in a inactive oligomer until a critical concentration of S105 can accumulate to trigger the lesion. The anti-holin prefers to form heteroligomers with the holin and therefore could function by titrating out the holin in a stoichiometric fashion during holin accumulation (Grundling *et al.*, 2000).

S105 is the best-studied holin; Cystine scanning mutagenesis established it has three TMDs (Grundling, Blasi, and Young, 2000a), sequencing gazing shows short loops connecting the TMDs, a small hydrophilic N-terminal domain, and a hydrophilic C-terminal tail rich in basic residues (Fig. 1-5). The lysis timing can be altered by single point mutations, the timing can be accelerated or delayed substantially. DSP crosslinking studies show that each mutant that is defective in hole formation are blocked at a certain step. S_{A48V} falls to form dimers, the initial step in hole formation.

A.

Lambda S

MKMPEKHD**LLAAILAAKEQGIGAILAFAMAY**LRGRYNG**GAFTKTVIDATMCAIIAWFIRDLL**DFA

              TM1   TM2

GLSSNLAYITSVFIGYIGTDSIGSLIKRF<u>GGHHHHGG</u>AAKKAGVEDGRNQ

    TM3   His Tag

B.
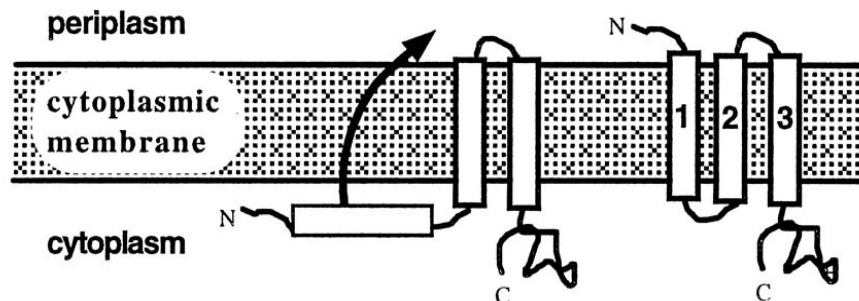
Figure 1-5. Lambda S sequence and topology.

A. The first methionine is indicated by (codes for S107 protein) and the second methionine (codes for S105 protein). Histidine sequence for purification is underlined. B. The primary topology of S105 (effector) and S107 (inhibitor) before triggering, S107 adopts the topology of S105 upon triggering. Transmembrane domains are in bold, underlined and labeled TM1,2,3.

S_{A52V}, S_{R33L} and S_{A23V}. It has been shown that the addition of energy poison causes premature triggering by the lambda holin (Blasi and Nam, et al. 1989). This result leads to a proton leakage model of how S could trigger and its relationship with the electrochemical potential of the inner membrane. This model was tested by anchoring *E.coli* by their flagella and expressing of the lambda lysis cassette. The proton motive force was monitored by the rotation of individual cells tethered by a single flagellum. The speed of rotation of tethered cells was directly proportional to the proton motive force up to at least -160 mV and probably through the entire physiological range.[A fully energized E. coli cell maintains its pmf at -180 to -200 mV (Harold and Maloney 2004)]. It was found that the membrane integrity was not compromised after the induction of holin synthesis until seconds before lysis, since the rotation of the cells did not change. These results further indicated that the holin was not poisoning the cell while building up to a critical level for hole formation. This would be expected since compromising the cell could slow down virion assembly. These results also suggested that perhaps lesion formation occurs when S reaches a critical concentration in the membrane.

A more detailed understanding of how S works will come from studying S in a purified system. To accomplish this task, a method of detecting lesion formation was created in which self quenching dye is loaded into liposomes and dye release can be followed by fluorescence (Smith, Struck, et al. 1998). A method to deliver functional S to the liposomes was created in this study, along with using bacteriorhodopsin to establish and control a membrane potential in liposomes (Fig. 1-6).
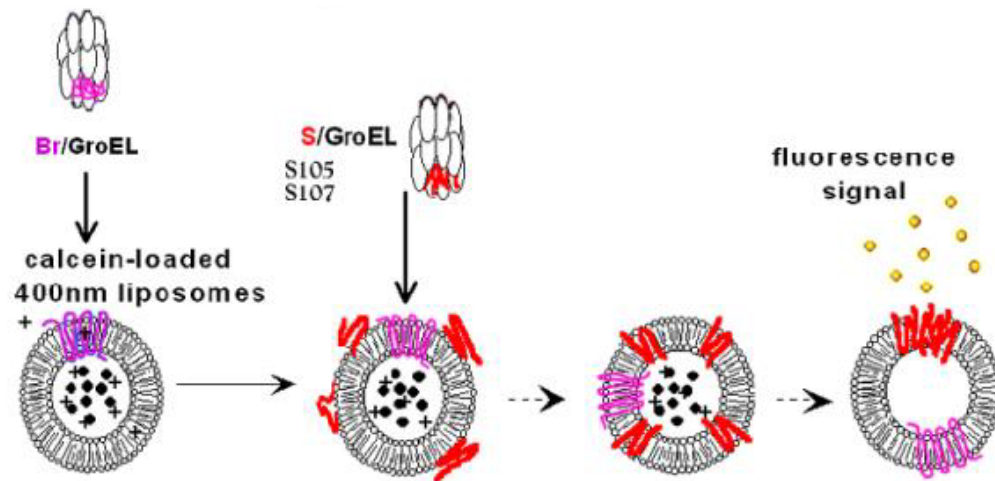
Figure 1-6. Assay to monitor holin activity *in vitro*.

GroEL solubilized BR was added to Calcein loaded liposomes, and a charge was created by activating BR at 560nm. When the dye was trapped it was self quenched and emitted low fluorescence, however when released by disruption of the membrane, the dye emitted high fluorescence signal. Next, S105 and/or S107 is added to the liposomes and hole formation can be followed by fluorescence resulting from dye release.

Bacteriorhodopsin

In order to reconstitute S into liposomes in the presence of a membrane potential, Bacteriorhodopsin was incorporated into liposomes. Bacteriorhodopsin (BR) is a compact molecular machine that pumps protons across a membrane powered by green sunlight. It is made by halophilic (salt loving) archaebacteria, found in high-temperature brine pools. They use sunlight to pump protons outwards across their cell membranes, making the inside significantly more alkaline than the outside. These protons are then allowed to flow back inwards through another protein, ATP synthase, building much of the ATP that powers the cell. BR is a seven-helical transmembrane protein with short interconnecting loops and a retinal co-factor. The helices (named A-G) tightly surround a retinal molecule that is covalently bound via a Schiff base to a conserved lysine (Lys-216) on helix G (Fig.1-7).

Bacteriorhodopsin has two half channels, one facing the extracellular side and the other facing the cytoplasm. The two halves are separated by the retinal bound G helix. Upon absorption of a light quantum photon (λmax at 568 nm), BR undergoes a series of photo interconversions initiated by the all-trans to 13-cis isomerization of the retinal, and has several spectral intermediates. A proton is transferred from the Schiff base to its counterion, asp-85. Concurrently, a proton is released on the extracellular side of the membrane, most probably from glu-204 with the help of arg-82. This leads to the formation of the strongly

blue-shifted (λmax around 410 nm) M intermediate. In the N intermediate, the Schiff base is reprotonated from asp-96, which is itself reprotonated from the cytoplasmic side of the membrane. In the O intermediate, the retinal reisomerizes to an all-trans configuration, while the glu-204 is reprotonated by asp-85 (Fig. 1-8).

Bacteriorhodopsin is an ideal proton pump for these experiments for several reasons. First, incorporation of only one protein is much simplier than protein pumps that require several subunits in order to function such as the F_1F_0 ATPase of *E.coli*. Second, functional BR has a purple tint due to the absorption properties of covalently bound retinal as stated above. Third, BR can be turned on or off during experiments by controlling the excitation wavelengths, enabling the experimenter to collapse or charge the membrane at any time during the experiment. This method will be very useful when studying proteins whose activity is correlated with a membrane potential, such as with S.
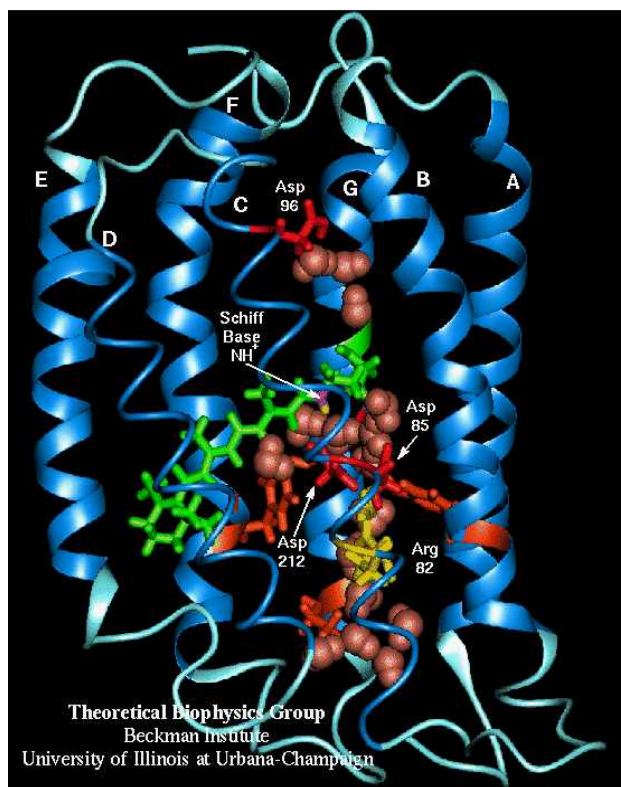
Figure 1-7. Crystal structure of retinal bound bacteriorhodopsin.

The seven helices (labeled A-G) tightly surround a retinal molecule that is covalently bound via a Schiff base to a conserved lysine on helix G. Important residues requires for proton pumping are labeled. Reproduced with permission from Theoretical and Computational Biophysics Group. Beckman Institute, 405 N. Mathews, Urbana, IL 61801, USA. All rights reserved. Available at http://www.ks.uiuc.edu/Research/newbr/
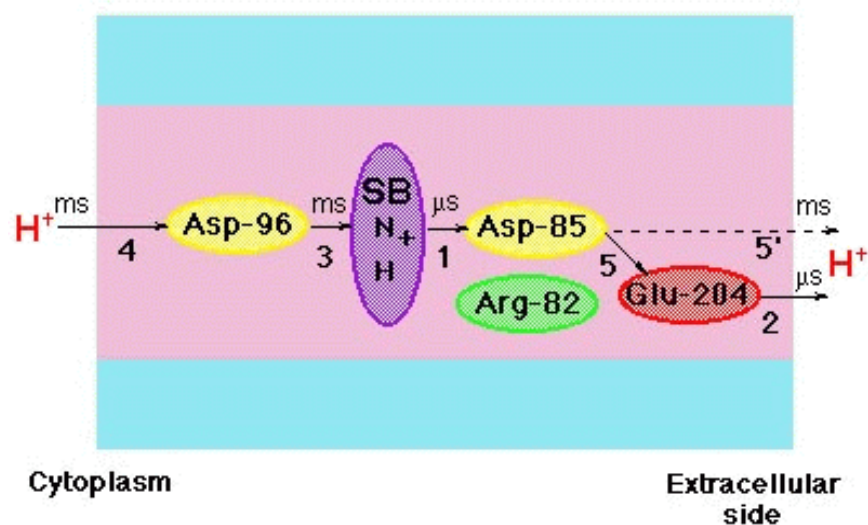
Figure 1-8. Key steps/residues involved in proton transfer in bacteriorhodopsin.

Absorption of light drives a proton across the cytoplasmic side to the extracellular side of a membrane. Residues that interact with the proton during this translocation are indicated. One photon translocates 1-2 protons across the membrane.

CHAPTER II

IN VITRO **CHARACTERIZATION OF THE LAMBDA HOLIN**

INTRODUCTION

Lambda uses the holin/endolysin cassette to lyse its host. The endolysin degrades the cell wall and the holin controls the timing of lysis by forming a lesion to allow the endolysin access to the cell wall or to activate the periplasmic endolysin by collapsing the membrane potential. S has genetically been studied in detail (Young and Blasi, 1995), with numerous point mutations altering the timing of lysis (Grundling, Blasi, and Young, 2000c) and illustrating that the only gene that controls lysis timing is the holin. The initial stages of developing a method to study S *in vitro* has been set. An over-expression vector has been designed to overproduce enough S to use in *in vitro* experiments (Hirai and Subramaniam, 2003). A histidine tag was inserted between positions 94 and 95 of S that did not change the lysis phenotype significantly and permitted purification by his tag chelating chromatography.

An important step *in vitro* reconstitution of a membrane protein was to find an environment in which the protein was stable; this usually requires detergent or a chaotrope. In order to study the activity of S, a liposome assay was developed (Hirai and Subramaniam, 2003). The assay involved self quenching dye loaded into liposomes and lesion formation was measured as a function of fluorescence release. In order to make this assay optimal, efficient delivery of S to the liposomes was a must. Delivery of S to lipid

bilayers is not understood. It is not even known whether S^{105} or S^{107}requires helper molecules to insert into the membrane *in vivo*, but depletion of the SecYEG translocon or poisoning of SecA function with azide does not affect the lysis function of S. S forms lesions in membranes, thus prevents using a common method of reconstituting membrane proteins by mixing detergent solubilized membrane protein and lipid together and dialyzing out the detergent. Instead Empigen BB solubilized S was dialyzed into 6M guanidine HCL. Initial results in a purified system, suggested that less than 1% of denatured S actually associated with the liposomes (Hirai and Subramaniam, 2003). This indicated in an artificial system, denatured S could spontaneously insert into membranes at low efficiency. In order to study the activity of S in artificial liposomes, the efficiency of delivery must improve. This study will optimize and report the initial results of this assay and suggest future work.

The S mutations such as S_{A52V} (which fails to trigger *in vivo*), S_{A55T} (a temperature sensitive allele), S_{A52G} (very early lysis) can be used as controls in the *in vitro* experiments (Wang, Smith, *et al.,* 2000) to ensure that the results are not an artifact of the system.

MATERIALS AND METHODS

Table 2-1. Strains and genotypes.

Strains		Genotype	
BL21 (DE3)		BF⁻ *omp*T *lon hsd*S gal (λ*imm*²¹*nin*5 *int*::*lac*I-*lac*UV5-T7gene*1*)	
MC4100		K12 F⁻ *ara*D139 Δ(*arg*F-*lac*) U169 *rps*L150 *rel*A1 *flb*B5301 *deo*C1 *pts*F25 *rbs*R	
XL1-Blue		K12 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F'*pro*AB *lac*IᑫZΔM15::Tn*10*(Tetʳ)]	
MZ1*			*his ilv rps*L *gal*K$_{am}$ pglΔ8 (*bio-uvr*B)ΔH1

* carries a defective lambda prophage. There are two major deletions. The first is the ΔB$_{am}$ in the P$_L$ operon and the other is ΔH1, which deletes the *cro* gene and all other lambda prophage genes to the right of *cro*. Only three lambda genes remain intact in this prophage: *N, rex,* and *c*I.

Lysis Curves

E.coli MC4100 cultures (table 2-1) with lambda lysogens (Δ SR) were grown to optical density of .2 and induced by heat shock for 15 minutes at 42°C and the growth curve was followed by optical density at 550nm.

Detergent

Empigen BB has a low CMC of 1.6-2.1mM; it can be dialyzed out and replaced with a chaotrope. Detergent can solubilize liposomes, thus releasing any contents of the liposomes. A standard curve of Empigen BB concentration versus fluorescence was made and Empigen BB could be detected as low as .01%. Over a six day period of dialysis, Empigen BB can be dialyzed out. This process can be completed to 24 hours by using Calbio-absorb beads outside of the dialysis bag as recommended by the manufacturer.

SDS PAGE

Protein fractions were analyzed by Tris Tricine 16.5% total SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to the method of Schägger and von Jagow. 16% TrisHCL Tricine pre-cast gels (NOVEX, San Diego, CA, Xcell Minicell) were also used routinely. Proteins were visualized by Coomassie Blue. Gels were fixed in 20% methanol, 5% glycerol and dried between two sheets of cellulose (Promega, Madison, WI).

Western blotting and immunodetection

For immunodectection of S protein, extracts and pre-stained molecular weight standards (Life Technologies Inc. (GIBCO BRL), Gaithersburg, MD) were resolved on 16.5% Tris Tricine gels (above). Proteins were then transferred to 0.11 micron nitrocellulose (Schleicher and Schuell Inc., Keene, NH) as follows.

All subsequent steps were done at room temperature with gentle shaking. The blot was incubated in blocking solution (2% non-fat powdered milk resuspended in 1×Tris-buffered Saline (TBS) (10mM TrisHCl pH7.6, 150mM NaCl)) for one hour. The primary antibody was diluted 1,000 fold into 0.6% milk in TBS prior to overnight incubation. The blot was rinsed once briefly with distilled water and twice for 10 minutes with 2% milk in TBS. Horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin (IgG) (Pierce chemical Co., Rockford, IL) was diluted 1,000 fold into 0.6% milk in TBS added to the blot and incubated for at least four hours. The blot was washed as previously described prior to development with substrate. The substrate was made immediately prior to use as follows. 12mg 4-chloro-1-naphthol were dissolved in 4 ml methanol. 20ml of

1×TBS were added followed by 10 µl 30% hydrogen peroxide. The solution was added to the blot and shaken gently until sufficient color developed, at which point the reaction was terminated by rinsing the blot with distilled water. Images of the blots and stained acrylamide gels were digitally recorded with a Hewlett-Packard ScanJet 6300C scanner. Quantitative analysis of gel and immunoblot data was performed using the image analysis program NIH Image.

Determination of protein concentration

Typical estimation of protein concentration of various samples was performed either by the Bio-Rad or DC Bio-Rad (detergent-compatible) Protein Assay (Bio-Rad Laboratories) or the BCA ™ (bicinchoninic acid) Protein Assay (Pierce) according to manufacturer's recommendations. In both cases bovine serum albumin (BSA) was used as a standard. Concentrations of purified samples of S were determined according to the method of Pace (Pace *et al*, 1995). Essentially, the molar absorption coefficient, ε, was measured and the concentration of protein in solution was determined from the Beer-Lambert Law: $A = \varepsilon l c$, where A is the absorbance at 280nm, ε is the molar absorption coefficient (M^{-1} cm^{-1}), l is the pathlength (centimeters (cm)), and c is the molar protein concentration. Samples of purified S protein were diluted into either buffer (1%Empigen, 10% glycerol, 20mM BES pH 7.0) or 6M GdnHCL and scanned in a Beckman Instruments Inc. (Fullerton, CA) DU-8 densitometer from 250 to 350 nm to check for light scattering. In the absence of light scattering, ε_{280} was calculated from:

$$\varepsilon_{280(6M\ GdnHCL)} = (\#Tryptophans)(5,500) + (\#Tyrosines)(1,490) + (\#Cystine)(125)$$

where S had one tryptophan, four tyrosine residues, and one cystine. The calculated

$\varepsilon_{280(6MGdnHCL)}$ for S105 was 11,460 $M^{-1}cm^{-1}$. According to this method of calculation the addition of an oligohistidine tag had no affect on the ε_{280}. The absorbance at 280nm in Guanidine HCL or detergent was divided by 11,460 $M^{-1}cm^{-1}$ and a pathlength of 1.0 cm to yield the concentration of S protein in buffer.

Holin purification

Oligohistidine–tagged holin and antiholin proteins were purified as described (Smith, Struck, et al. 1998), with some modifications. Membranes from cells producing these proteins were subjected to differential solubilization in 1% EBB, 10% glycerol, 20mM BES, 0.5M NaCl, 35 mM $MgCl_2$ pH 8.0 for 2 to 18 hours at 37C with shaking. Insoluble material was removed by centrifugation at 100,000g for 45 minutes and the soluble extract was filtered through a 22 mm syringe filter (Genemate Bioexpress) and loaded onto a Hitrap Chelating HP nitrilo-triacetic acid column (1 ml) charged with $CoCl_2$ and equilibrated with 1% EBB, 20mM BES, 0.5M NaCl, 10% glycerol, pH 7.6. After loading, the column was washed with 1% EBB, 20mM BES, pH 7.6 and eluted at a flow rate of 0.5ml/minute with a pH gradient from pH 7.6 to pH 2.5, using 1% EBB, 20mM sodium acetate, 0.5M NaCl as the limit buffer. Oligohistidine-tagged proteins were eluted with a pH gradient. Eluted protein fractions were neutralized with 0.1 M NaOH.

Immuno precipitations

E.coli MC4100 cultures with lambda lysogen (S105-94H,R, Rz,Rz1) induced by

heat shock at 42°C, and 16mls of culture were taken at 25 minutes and 40 minutes. A control was used in which the lambda holin, S, has an amber mutation at position 7 (S_{am7}). The cells were centrifuged in the presence of 5mM nickel and resuspended in buffer containing protease inhibitors. The cells were then lysed by lysozyme and EDTA, or chloroform and EDTA, or French Press. The lysate was spun down at 5000rpm for 30 seconds to remove any non-lysed cells. GroEL antibodies were added to the cell lysates and put on a roller for one hour. Secondary antibodies with an iron cluster (Pierce MagnaBind beads) were added to the cell lysates per manufacturer's recommendations and the solution again was put on a roller for one hour at 30°C. The lysates were exposed to a magnetic field and the antibodies were pulled to the side and washed twice. The pull downs were then re-suspended in sample buffer and western blotted.

Purification of GroEL

GroEL was expressed from the polycistronic *groES-groEL* mRNA transcribed by the lambda P_L promoter in the pRE1 expression vector received from Kamireddi et al. *E. coli* strain MZ1 carrying the pRE1-*groESL* recombinant plasmid was grown in 3 liters of LB medium containing 1mM ampicillin at 30°C to an A_{550} of 0.4. The temperature was raised to 42°C and the culture was shaken for 5 to 6 hours. The cells were collected by centrifugation at 10,000g for 15 minutes and resuspended in French press buffer (50mM Tris-HCL, pH8.0, 1mM EDTA, 1mM DTT, and .1mM PMSF) and ruptured by passage through a French press at 16,000 psi. The French press cell lysate was centrifuged at 100,000g for 1 hour. The supernatant was passed over an Amersham-Pharmacia Biotech

5mL High-trap Q column equilibrated in french press buffer on an AKTA FPLC. GroEL in the high salt elution fraction was precipitated with ammonium sulfate (50% saturation), equilibrated for 1 hour and the precipitate was collected by centrifugation at 12,000g for 30 minutes. The ammonium sulfate precipitate was dissolved in 12 mL of french press buffer and GroEL was purified by gel-filtration chromatography on a Superdex 200 column (Amersham Pharmecia Biotech) equilibrated with french press buffer. GroEL was eluted in the void volume and pure fractions, as judged by SDS-PAGE analysis, were pooled.

Purification of bacteriorhodopsin

Halobacterium halobium (mutant R1) cells were grown in Doolittle media (in 1L: NaCl, 250g; MgSO$_4$,20g; trisodium citrate 3g; KCL, 2g; (Oxoid Bacteriological Peptone, 10g; or Bacto Yeast Extract (Difco), 3g; and Bacto Tryptone (Difco), 5g)) for five days and collected by centrifugation at 10,000g for 10 minutes. The cells were resuspended in 1 L basal salt (NaCl, 250g; MgSO$_4$, 20g; NaCitracte, 3g; KCL, 2g; Dnase, 5mg) and were dialyzed overnight against 0.1M NaCl. The solution was a clear red lysate. The cells were centrifuged at 40,000g for 40 minutes and the reddish purple sediment was resuspended in 300ml of 0.1M NaCl. The centrifugation/resuspension step was repeated until the lysate was almost colorless. The pellet was resuspend in 6-10ml deionized water and washed/centrifuged again until the supernatant was almost colorless. The resuspension was layered over a linear 30 to 50% sucrose gradient with SW27 rotor at 100,000g for 17 hours at 15°C until it reached an equilibrium at a density of 1.18g/cm^3. The purple band was collected and the sucrose was removed by dilution to 300ml of water and centrifugation was repeated at 50,000g for 30 minutes.

Delipidation of bacteriorhodopsin

The suspension of purple membrane in water was centrifuged at 8,000g for 10 minutes and the pellet was re-suspended in 0.5ml (vol/vol) Triton X-100/0.1M sodium acetate, pH 5.0 and the mixture was allowed to stand, with occasional vigorous agitation (vortex), in the dark at room temperature for two days. The solution was then centrifuged at 8,000g for 1 minute and the supernatant was applied to an Amersham Pharmecia Biotech Superdex 200 column that had been pre-equilibrated with 0.01M Tris-HCl/0.15 M NaCl/ 0.25% deoxycholic acid/0.025% NaN_3 titrated to pH 8.0 with NaOH. The column was eluted in the dark at 4°C with the above equilibration buffer. Fractions containing delipidated bacteriorhodopsin were rapidly frozen and stored in the dark at -20°C.

GroEL solubilization of membrane proteins

To form complexes between membrane proteins and GroEL, 800 ml of 1% EBB, 20mM BES, 0.5M NaCl, pH 7.6 was placed in a tube and 100 ml of a GroEL solution in the same buffer was added. Finally, 100 ml of membrane protein in the same buffer was added. At each step, the solution was mixed by pipet. For most experiments, the final concentration of GroEL was 100 mg/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 mls of 20mM BES, 0.5M NaCl, pH 7.6, supplemented with Calbiosorb Bio-Beads. Buffer and Bio-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 BSA. In these experiments,

the efficacy of detergent removal was assessed using calcein-loaded liposomes as previously described (Smith, Struck, et al. 1998). In all cases, detergent was reduced to less than 10% of its critical micellar concentration.

Circular dichroism (CD)

Purified samples of S protein were analyzed on an Aviv Associates Inc. Model 62DS CD spectrometer at wavelengths from 195 to 300nm in a 0.1 cm pathlength cuvette at a final concentration of 15 to 25 μM in either 1% octyl-glucoside, deoxycholate, Triton X-100 or Empigen BB. Scans were measured in millidegrees (m°) and converted to mean residue ellipticity by the equation,

$$[\theta] = (\Delta m° \times 100)/(n \times c \times l)$$

where $[\theta]$ is the mean residue ellipticity, $\Delta m°$ is the m°(sample)- m°(blank), n is the number of residues in the peptide, c is the concentration of the protein in solution (mM), and l is the pathlength.

Marker release assay

Marker release experiments were performed on SLM 8100 Fluorescence spectrophotometers (Spectronic Instruments Inc. Rochester, NY). Marker release assays were performed in 2.5ml glass cuvettes with all four sides polished. 2.0ml total volume TBS was added to a cuvette with 40μl of liposomes (ca. 10^{12} liposomes). The samples were excited at a wavelength of 490nm and emission was monitored at 520nm. Baseline, or 0% relative fluorescence was defined as the signal acquired upon addition of the desired amount of buffer, 6M GdnHCL in TBS. Total release, and therefore 100% relative fluorescence,

was obtained by adding 10µl 10% Triton X-100. Protein (2 to 15µg) was added to initiate the assay.

Electron microscopy

GroEL and GroEL plus membrane protein at 0.1 mg protein/ml were negatively stained using a 2% (w/v) aqueous solution of uranyl acetate and omitting any fixation steps. Specimens were observed in a Zeiss 10C transmission electron microscope operated at 80 kV and were images recorded at calibrated magnifications (35,500x and 27,000x). Selected micrographs were digitized using a Leafscan 45 at 20 mm increments corresponding to 0.56 and 0.74 nm/pixel, respectively at the specimen level, and processed using the IMAGIC 5 and EMAN (Ludtke, Baldwin, et al. 1999). Approximately 700 particles of the top view of GroEL from both samples were selected for analysis. After translational alignment particles were subjected to multivariate statistical analysis (MSA) to yield class averages. A difference map was calculated by subtracting densities of rotationally aligned class averages from the two samples.

RESULTS

S localizes to the inner membrane when turned on during late gene expression. As with other membrane proteins, certain aspects of purification had to be implemented, such as addition of detergent, to maintain solubility. S is lethal in small doses to *E.coli,* yet overexpression from a pET vector resulted in mg quantities of protein from a liter of culture, thus a histidine tag was placed on the protein and the gene was inserted into a T7 system. Surprisingly, rapid overproduction of the holin produces milligram quantities (Smith and

Table 2-2. Detergent solubility of S.

Detergent	MW (anhydrous)	CMC (mM)	Aggregation #	Estimated %S extracted and solubilized by detergent
n-Octyl-β-D-glucopyranoside (OG)	292.4	20-25	84	60% extracted purified S precipitates in less than an hour in OG
Triton X-100	-	0.2-0.9	100-155	70% extracted detergent removal ineffectual by dialysis.
MEGA-9 Ultrol Grade	335.5	19-25	-	20% extracted
n-Heptyl-β-D-thioglucopyranoside	278.3	79	-	50% extracted S precipitates
Genapol X-80 Protein Grade	553	0.06-0.15	-	50% extracted detergent removal ineffectual by dialysis
Tween 20	-	0.059	-	50% extracted detergent removal ineffectual by dialysis
Cholic Acid	430.6	9-15	2.0	70% extracted detergent precipitates below pH 6.0
Taurocholic Acid	537.7	3-11		50% extracted S rapidly precipitates
Deoxycholic Acid, Sodium Salt ULTROL Grade	414.6	4-8	3-12	70% extracted detergent precipitates below pH 6.0
CHAPS	614.9	6-10	4-14	70% extracted S rapidly precipitates
Zwittergent 3-10	307.6	25-40	41	50% extracted S rapidly precipitates
Zwittergent 3-12	335.6	2-4	55	20% extracted
Empigen BB	272.0	1.6-2.1	65	90% extracted S stable for months

Estimated % S solubilized by detergent: S extracted in membrane extraction buffer was compared with S in detergent insoluble material against total amount of S in French press buffer on an SDS Commassie blue gel (samples were adjusted to equal volume). Separate controls of dialyzed detergent alone was tested using fluorescence release of detergent sensitive liposomes.

Young, 1998). Purification with a chelating column into detergent was possible, but presented several problems. First, S became highly unstable in Triton X-100, Octyl-Glucoside and several other detergents. Stability ranged from several minutes to hours in solution depending on the detergent, making accurate *in vitro* assays very problematic. Second, S in Octyl-glucoside showed only 46 amino acids in alpha helical formation (Smith and Young, 1998), which would be enough amino acids for two transmembrane domains. *In vivo* research (Grundling, Blasi, and Young, 2000a) suggests that at least three transmembrane domains exist. Third, The only way to prevent S from falling out of solution was to dialyze it into a chaotrope, causing further problems with some *in vitro*. In this study a new detergent was used to purify S. Empigen-BB was a zwitterionic detergent that has a CMC of 1.6-2.1mM, it could be dialyzed (Fig. 2-1) and it did not absorb at wavelengths between 200-300nm, making it ideal for CD spectra and determining concentration of S at 280nm. S in Empigen BB showed 62 amino acids in alpha-helix conformation on CD spectra (Fig 2-3), enough for three transmembrane domains. Using Empigen BB in the purification scheme also doubled the amount of S that comes off the column. S can also be successfully dialyzed into guanidine and shown to mimic *in vivo* characteristics when placed into self-quenching dye filled liposomes.

The first significant improvement in purification was the implementation of Empigen BB for the extraction and solubility of S. Previously, Triton X-100 was used to extract the protein and n-Octyl-β-D-Glucopyranoside (OG) was substituted during the

binding and delution of the protein, so the protein was in a detergent that could be dialyzed (OG). The protein remained stable only for a few minutes after elusion, preventing any major biochemical characterization of the protein. The extraction of S from the *E.coli* inner membrane by Empigen BB was more effective than extraction of S by Triton X-100 as determined by Coomassie blue stained gels (Table 2-2). Empigen BB was a dialyzable detergent as determined by fluorescence of detergent sensitive dye loaded liposomes (Fig 2-1) and eliminated the need for multiple detergents for extraction, purification and solubility of S105-94H. Over-expression and purification of S yielded from 0.5mg/1L up to 12mg/1L of culture depending on allele. S was determined to be 99% pure by coomassie blue (Fig. 2-2) and silver staining.

Secondary structure of the lambda holin

The CD spectrum of S in Empigen BB gave approximately 54% alpha helical content at $[\theta]=222$nm. Which is roughly 62 amino acids, enough to form three transmembrane domains. This number was consistent with previous *in vivo* results (Fig. 2-3)(Grundling, Blasi, and Young, 2000a), unlike the unstable S^{94H} in OG, which showed only two transmembrane domains (Smith, Struck, et al. 1998). The purification protocol was applied to the following S alternate function mutants; S_{A52V}, S_{A55T}, S_{A52G} and the S inhibitor S107.
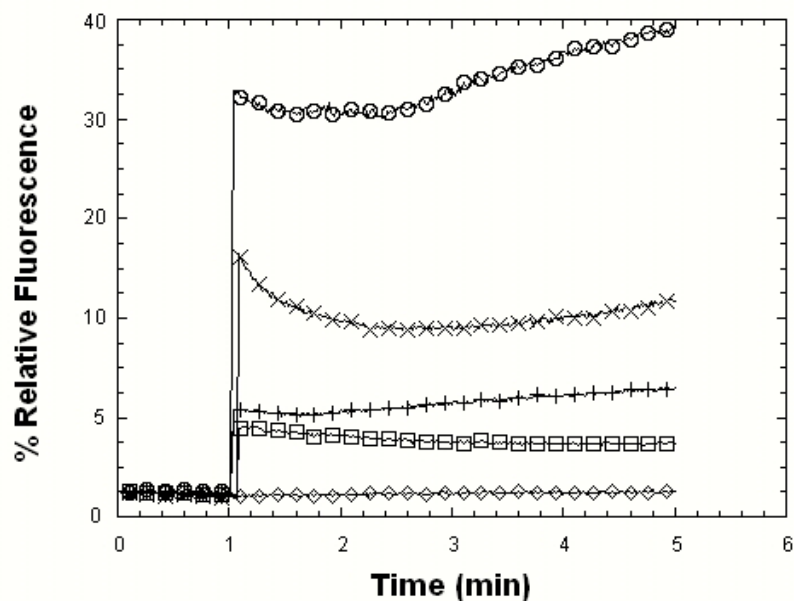
Figure 2-1. Empigen BB can be removed from samples by extended dialysis.

The progress of detergent removal can be monitored by dye loaded liposomes. At various times during dialysis; (circle) dialysis after 1 day, (X) 2 days, (+) 3 days, (square) 4 days, (diamond) > 6 days. Samples are removed and liposomes are added under standard dye release assays and fluorescence is monitored as described in *Materials and Methods*. Fluorescence release resulted from different Empigen BB concentrations were used to make a standard curve. This method can detect Empigen BB concentrations as low as .01% . Detergent removal was accelerated by using Cal biochem biobeads.

Lanes

MW 1 2 3
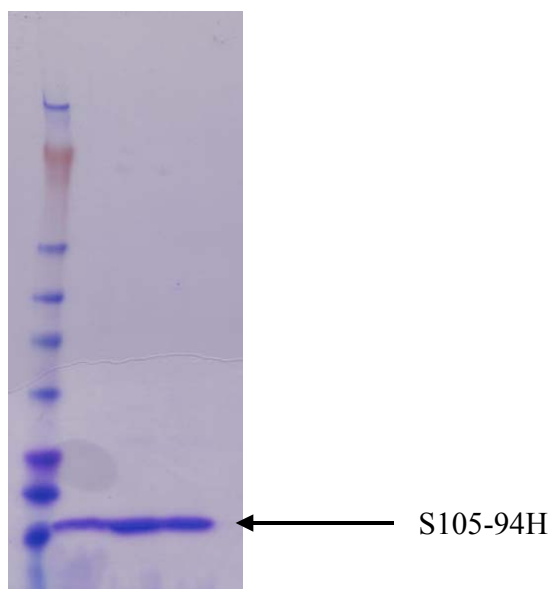
→ S105-94H

Figure 2-2. S105 can be purified using a histidine tag and chelating chromatography.

Lanes 1 through 3 are Coomassie blue stained eluted fractions of S105-94H from a cobalt chelating column eluted with pH gradient.
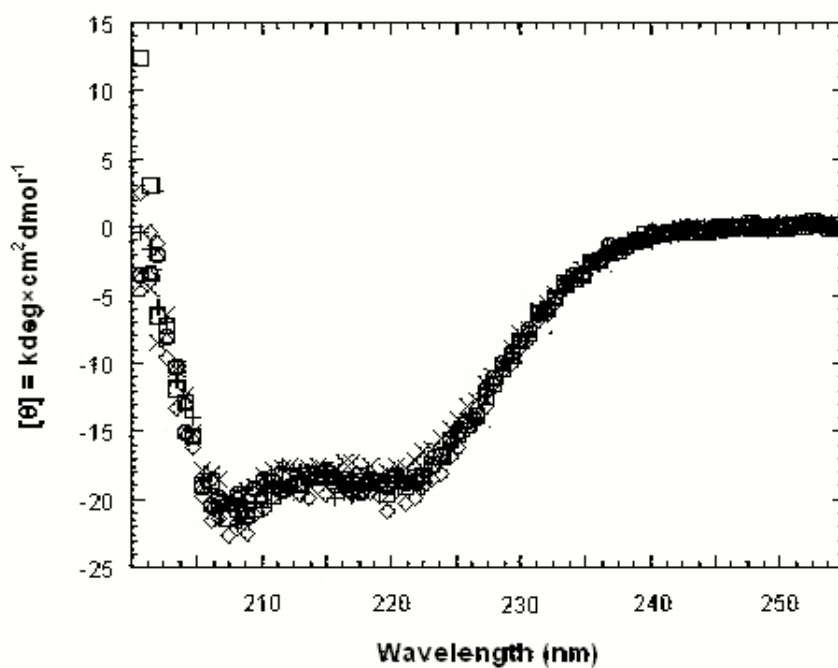
Figure 2-3. Circular dichroism (CD).

CD spectra of various alleles of S. ($\times$) S105$_{A55T}$-94Hat 42C, (square) S105$_{A55T}$-94H at 30C, (diamond) S107-94H, (triangle)S105-94H at 30C, (circle)S105-94Hat 42C, (+)S105$_{A52V}$-94H.

The circular dichroism spectrum of S105, S107 inhibitor, S_{A52V} non-functional allele and

S_{a52t} temperature sensitive allele done at the permissive and non-permissive temperature,

also showed enough alpha helical content to form three transmembrane domains, suggesting

that the different mutant phenotypes were not attributed to the secondary structure that can

detected by CD.

N-terminal translocation of the lambda holin

The only thing difference between the S105 (effector) and S107 (inhibitor) proteins

was the two additional amino acids with a net positive charge. Due to the additional positive

charge, could the translocation of the n-terminus of S107 (inhibitor) and S105 (effector) be

different, thus explaining their differing roles in lysis? The topology of S105 and S107 has

been studied using cysteine scanning mutagenesis and n-terminal signal sequence functional

assays. These studies showed two things; one, three sets of residues are protected by the

membrane, indicating three transmembrane domains. The second was that the addition of

a N-terminal signal sequence showed Lep-dependent function, suggesting the N-terminus

required transporting for lesion formation. Though no data has been shown that the n-

terminus of S105 and S107 vary in translocation across the inner membrane of *E.coli*.

E.coli membrane proteins are deformylated depending on their rate of translocation. *E.coli*

peptide deformylase was an essential metalloenzyme required for the removal of the formyl

group at the N-terminus of nascent polypeptide chains in eubacteria (Rajagopalan, Datta,

and Pei, 1997). This reaction only occurs in the cytoplasm after an unknown period of time

after the peptide is released from the ribosome. Depending on the rate of translocation of

the n-terminus of S105 and S107, the pattern of deformylation of holin and anti-holin may

not be the same. The S105 and S107 were purified and sequenced using edman degradation. Edman degradation is a cyclic degradation of peptides based on the reaction of phenylisothiocyanate with the free amino group of the n-terminal residue such that amino acids are removed one at a time and identified as their phenylthiohydantoin derivatives (Edman, 1950). Proteins that translocate their n-terminus quickly through the inner membrane would still retain their formyl group and would be blocked and hence, unable to be sequenced. However, if the n-terminus was retarded in the cytoplasm, then deformylase would remove the formyl group and the protein could be sequenced. S107 was sequencable, as were S105 with the first transmembrane domain deleted and the class II holin S^{21}, both proteins were predicted to have their n-terminus in the cytoplasm. The S105 protein however, was blocked and required the treatment of purified peptide deformylase (Rajagopalan, Datta, and Pei, 1997) in order for the S105 protein to be sequenced (Fig. 2-5). These results suggest that the kinetic "flipping" of the amino terminus of S107 and S105 are different and shows that the S107 amino terminus resides in the cytoplasm long enough to be deformylated, which does not occur with S105. Two other proteins (S^{21-68} and $S105^{\Delta TM1}$) that should have, that also have their amino termini in the cytoplasm, were both sequenced.

These results support the model that the S107 protein retains its n-terminus in the cytoplasm and either before or after triggering, the n-terminus is translocated across the membrane, in affect adopting the topology of the effector holin S105. The fact that de-formylation of the n-terminus of proteins from $E.coli$ only occurs in the cytoplasm, it would depend on the time it takes to translocate the n-terminus of proteins such as S107 and S105,

whether they are de-formylated or not. The two protein products have identical sequences except for the two additional amino terminal amino acids on S107, a methionine and lysine. The additional positive charges on S107 could impede the translocation of the amino terminal transmembrane domain through the inner membrane, requiring the collapse of the proton motive force before making translocation of the first transmembrane domain energetically favorable (Graschopf and Blasi, 1999). S105 would not have this problem, because without the additional charges the amino terminus should automatically insert into the membrane. The delayed translocation of the n-terminus was not unique, as leader peptidase has been shown to also involve a similar mechanism (Houben *et al.*, 2000). If the amino terminus resides in the cytoplasm for an extended period of time, then it can be deformylated as seen with S107, S^{21-68} and $S105^{\Delta TM1}$. When the amino terminus is rapidly transported to the periplasm, then the protein should still retain its formyl group, as seen with S105.
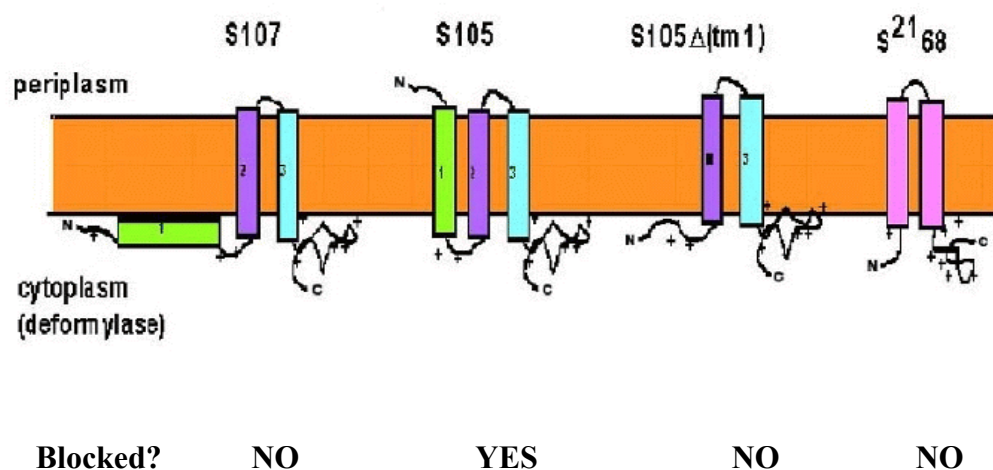
Figure 2-4. The topologies of S107, S105 $S^{21\text{-}68}$ and S105$^{\Delta TM1}$.

Each purified protein was sequencable by Edman degradation as described in Material and Methods. Proteins that were blocked were treated with purified deformylase and methionine amino peptidase and sequenced again to show the block was due to the protein still retaining its formyl group.

Effect of lipid composition on S function *in vitro*

The assay has improved by introduction of Empigen BB and by altering the lipid composition. In this assay, purified S protein, dialyzed into a detergent-free chaotropic solution, was diluted into an aqueous suspension of liposomes loaded with calcein. The permeabilization ability of S was monitored by fluorescence caused by the release of calcein, which is self-quenched within the liposomes. The kinetics of dye release caused by the addition of S varied with the composition of the liposomes. Liposomes made from 100% phosphatidylcholine showed lower overall amounts of dye released; yet the initial rate of release seems to be faster. However, S in liposomes made from 70% phosphatidylcholine and 30% phosphatidylglycerol, which resembled the overall charge of the *E. coli* cytoplasmic membrane, showed greater amounts of dye release overall but appeared to have an initial lag time (Fig. 2-5). By exploiting the improved purification and assay for the lambda holin, several results were obtained. First, that S produced from a lysis-defective missense allele, S_{A52V}, was inactive in dye release. Second, that S produced from a *ts* allele of *S*, S_{A55T}, releases dye at the permissive temperature (30C) but not the non-permissive temperature (42C), in contrast to the *wt* protein, which was active at both temperatures (Fig. 2-6). These results showed that the *in-vitro* assay reflected the *in vivo* phenotype of the different S alleles and that dye release was not an artifact of the assay.
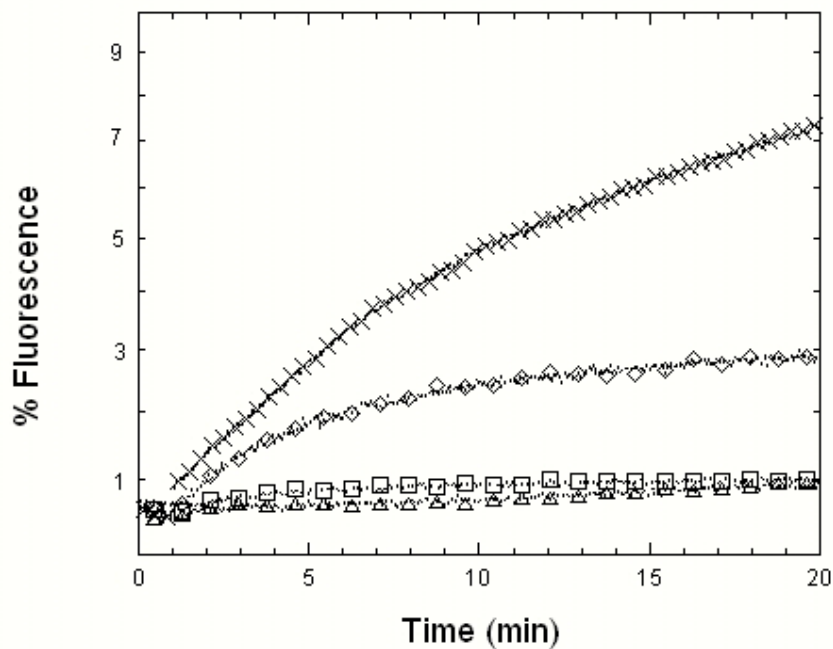
Figure 2-5. Dye release of S solubilized in 6M guanidine HCL.

The addition of anionic lipids to the composition of the liposomes increased the amount of dye release upon addition of a fixed amount of S. ($\times$) S105-94H added to 70%PC/30% PG vesicles at 12µg/ml. (circle) S105-94H added to 100% PC vesicles.(square) S105$_{A52V}$-94H added to 70%PC/30%PG at 40µg/ml. (diamond) S105$_{A52V}$-94H added to 100% PC vesicles at 40µg/ml.
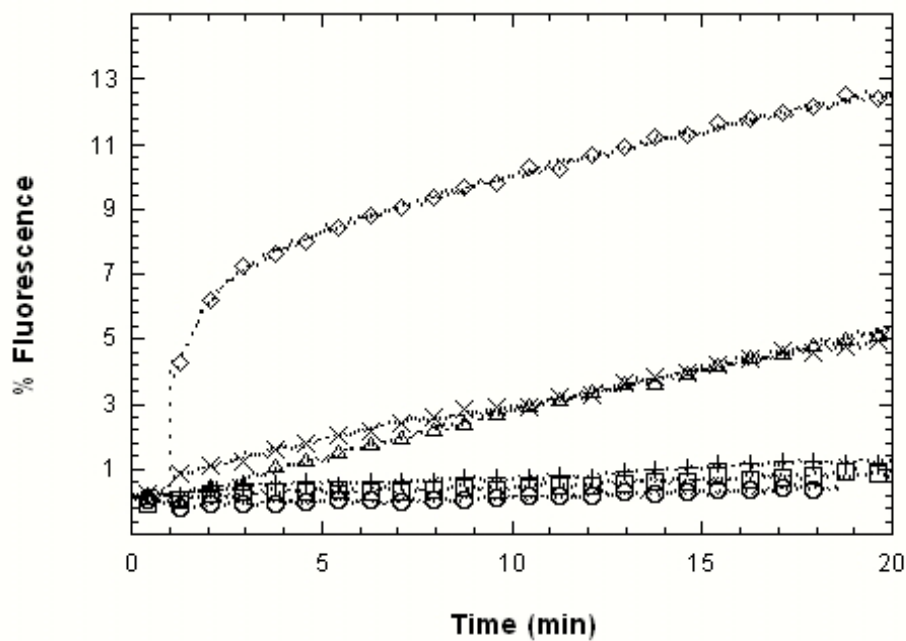
Figure 2-6. Dye release of various S alleles

The *in vitro* activity of S105, S105^{A52V}, S105^{A55T} reflects their *in vivo* phenotype. (diamond) S105-94H at 12μg/ml, (×) S107 at 12μg/ml, (triangle)S105$_{A55T}$-94H at 9μg/ml at 30C, (+) S105$_{A55T}$-94H at 9μg/ml at 42C, (square)S105$_{A52V}$-94H at 30μg/ml, (circle)buffer.

GroEL delivery of different S alleles to preformed lipid vesicles

A major problem still existed with this *in-vitro* assay. The sensitivity of the liposomes requireed S to be delivered from a chaotrope. S being diluted out of a chaotrope into an aqueous buffer resulted in 99% of the protein precipitating. This maked deliver of the holin to liposomes not very efficient.   As detailed in Chapter III, GroEL can solubilize and deliver functional S to liposomes in the absence of detergent. The overall fluorescence was greater when the same concentration of S added to liposomes was delivered by GroEL instead of coming out of a chaotrope (Fig. 2-7).

S alleles diluted out of GroEL also reflect their *in vivo* phenotypes, such as the ability of histidine tagged S alleles ability to form holes can be blocked by the presence of Ni^{++}(Fig. 2-8), which presumably binds the oligo-histidine tag in the C-terminal domain, and subsequently activated by addition of a chelator. The latter achievement indicates that the processes of binding and insertion may be biochemically distinct from the process of hole-formation.
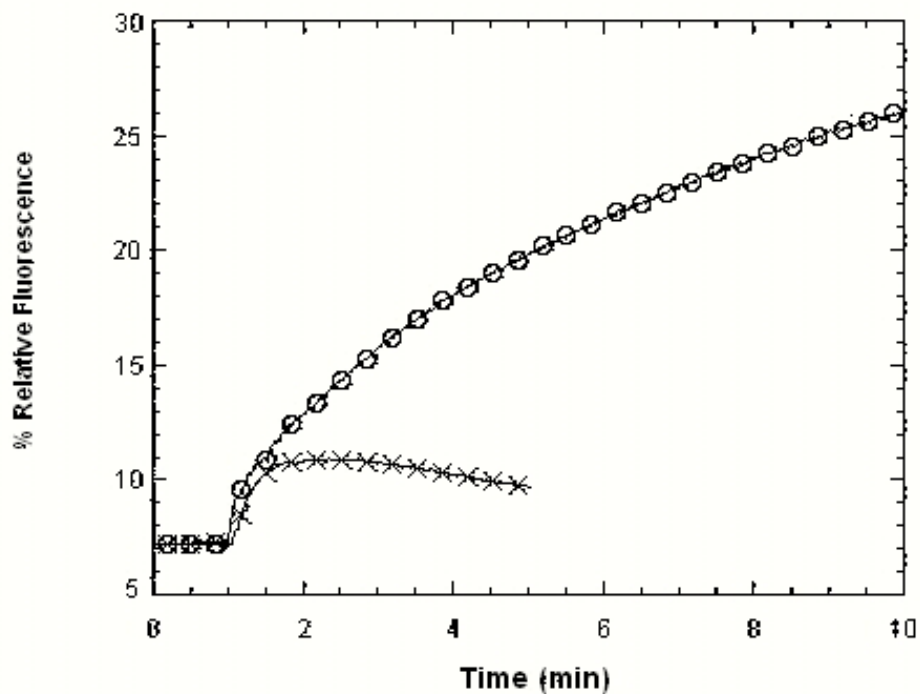
Figure 2-7. S was solubilized either by GroEL or 6M guanidine HCL.

Each sample was then diluted into calcein loaded liposomes, while following the fluorescence of calcein. (o) GroEL delivered S105-94H at 5μg/ml. (×) S105-94H at 5μg/ml diluted out of 6M Guanidine HCL.
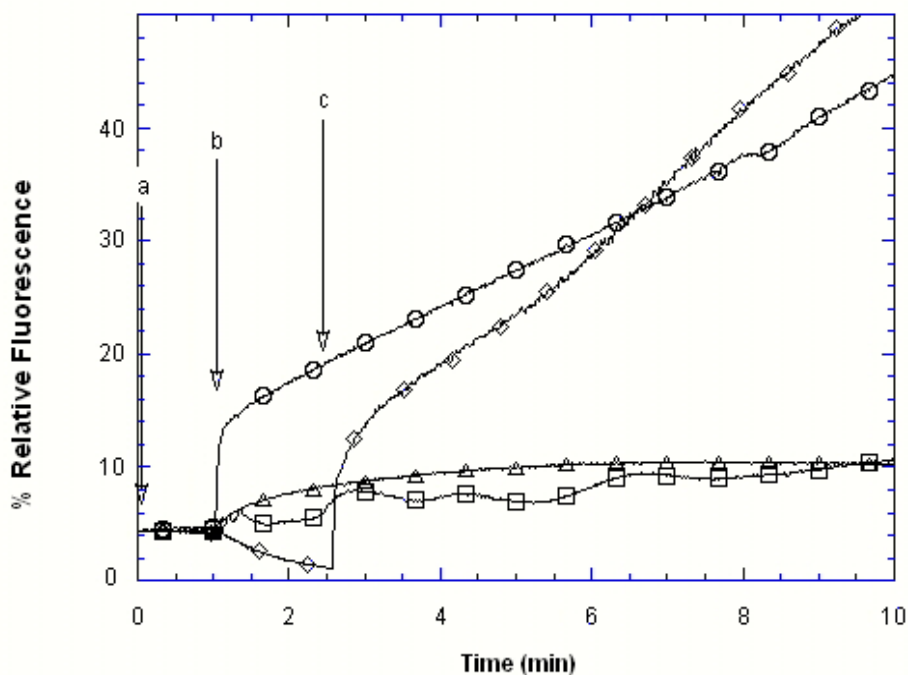
Figure 2-8. Nickel inhibits his tagged S *in vitro*.

The following components were added to calcein loaded liposomes. (a) addition of 1 mM nickel sulfate to (square)S105$_{A52V}$-94H at 30μg/ml, (diamond) S105-94H at 10μg/ml samples, (b) addition of (circle) S105-94H at 10μg/ml, (triangle)S105$_{A52V}$-94H at 30μg/ml, (square)S105$_{A52V}$-94H at 30μg/ml, (diamond) S105-94H at 10μg/ml and (c) addition of 10 mM EDTA to all samples.
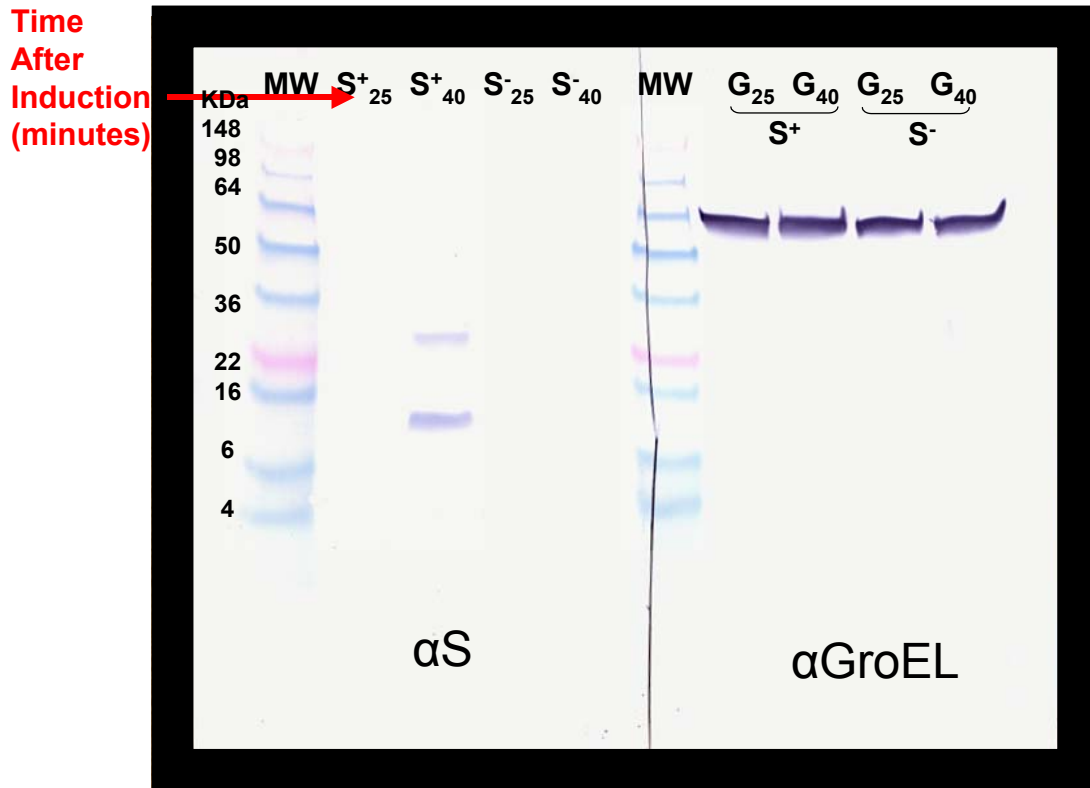
Figure 2-9. GroEL immunoprecipitation *in vivo*

Cells were harvested at either twenty five minutes or forty minutes after induction (lambda lysis time is fifty minutes). The cell lysates were subjected to immunoprecipitations by α-GroEL, fractionated by a SDS-PAGE and western blotted with α-S or α-GroEL.

S associates with GroEL *in vivo*

To determine if GroEL associated with S *in vivo*, *E.coli* carrying a lambda prophage was induced and the cells were spun down, lysed and the lysates were treated with either GroEL or S antibody overnight. When the primary antibody in the pull down was GroEL, the GroEL western blot and S western blot both produced a band corresponding to the right molecular weight for each protein. S was immunoprecipitated with GroEL when GroEL antibodies where present, indicating that GroEL and S are associated in the cell (Fig. 2-9). These results suggest that GroEL may play an *in vivo* role in holin solubility in the cytoplasm and/or translocation of the holin to the inner membrane. Immunoprecipitation experiments using S antibody failed to pull down GroEL or even S itself. This could be due to the conformation of the complex / environment S was in or the quality of the S antibody.

A second way to test if GroEL interacts or translocates holins to the inner membrane is to determine the effect of GroEL overexpression on the kinetics of lysis (Young, Wang, and Roof, 2000). An early lysis time could indicate that GroEL does deliver S to the membrane and it is a rate limiting step due to the amount of GroEL available for S. A

delayed lysis timing could be due to additional GroEL altering the equilibrium between S in GroEL and S in the membrane. No change in lysis timing could be a result of GroEL not being a rate limited step.

Lambda deleted of its lysis genes has been used to determine if genes from other phages are lysis genes. Many holins and endolysins have been clones into lambda and expressed. Using this technology, holins and endolysins from other phages can also be tested by placing them under the control of the lambda late promoter, PR' on a plasmid; where the lytic genes can be transactivated by inducing a temperature-sensitive lambda lysogen that is ΔSR.

The results varied depending on the phage lysis cassette used. The lambda lysis phenotype changed only slightly with an excess of GroEL, actually delaying lysis for five minutes (Fig. 2-10) . Similar results could be seen with the class II holin S^{21} (Fig.2-11), where again lysis timing is delayed at least ten minutes. Whereas a large change in lysis could be viewed with the P2 holin lydA (Fig. 2-12), in which an increase in lysis timing up to forty minutes could be seen.
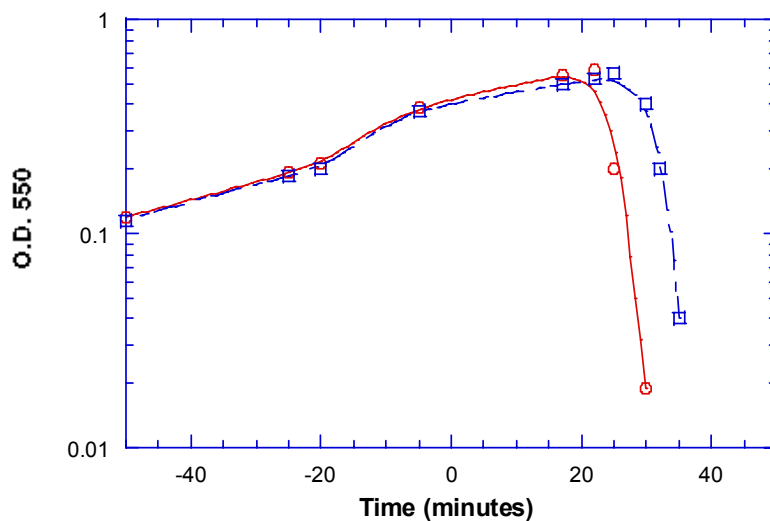
Figure 2-10. Overproduction of GroEL on lysis timing by lambda holin.

A plasmid (pRE) carrying *groEL* under T7 control is transformed into *E.coli* carrying a lambda prophage. The lysis curve of lambda lysis cassette of R (endolysin), S105 (holin) and S107(antiholin) (square). The lysis curve of lambda lysis cassette of R (endolysin), S105 (holin) and S107(antiholin) in the presence of overexpressed GroEL from a pRE plasmid (circle).
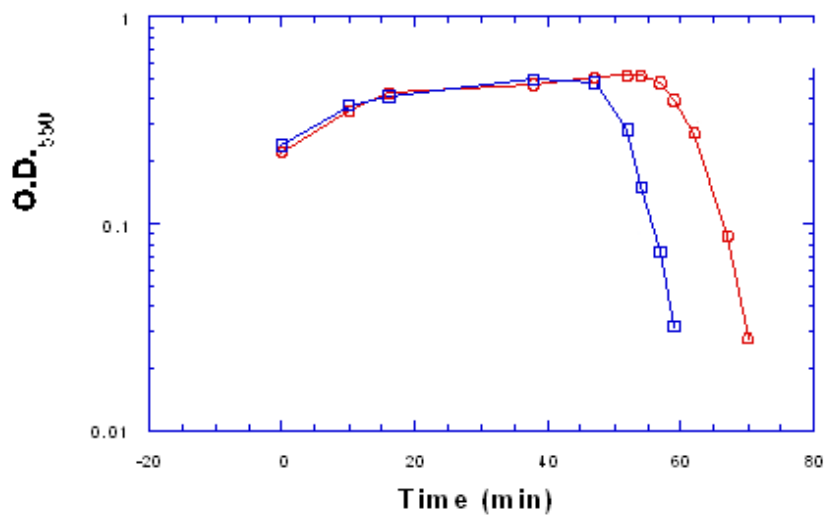
Figure 2-11. Overproduction of GroEL on lysis timing by phage 21 holin.

A plasmid (pRE) carrying *groEL* under T7 control is transformed into *E.coli* carrying a lambda prophage. The prophage's lysis genes are replaced with R^{21} (endolysin), S^{68} (holin) and S^{78} (antiholin) of the bacteriophage S21. The lysis curve of S21 lysis cassette in this context (square). The lysis curve of S21 lysis cassette in the presence of GroEL overexpressed from a pRE plasmid (circle).

A.
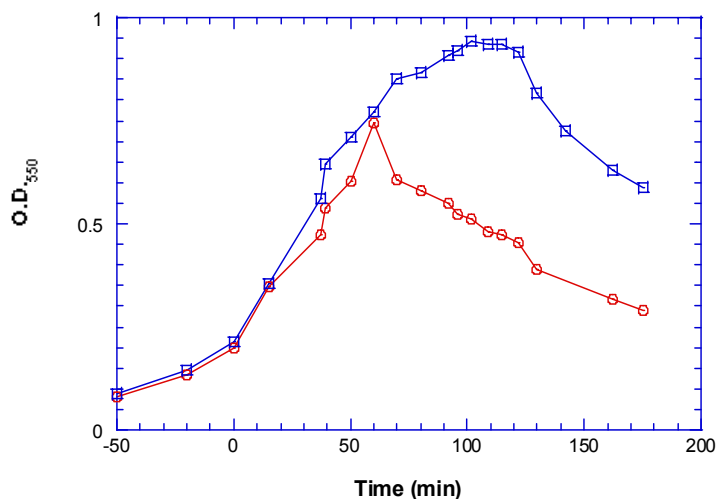
B.
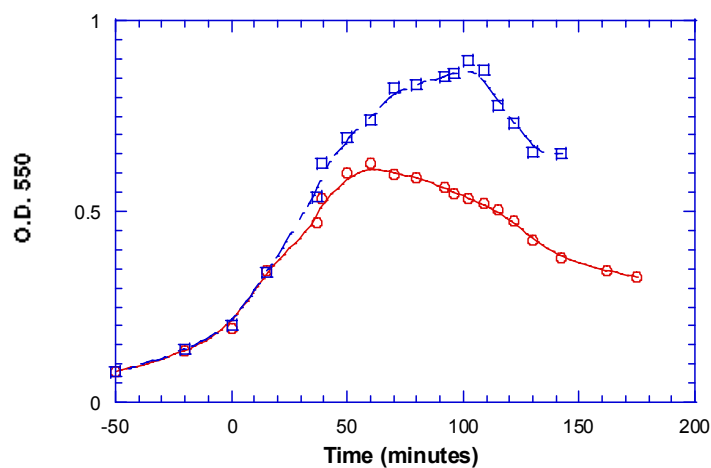
Figure 2-12. Overproduction of GroEL on lysis timing by P1 holin.

A plasmid (pRE) carrying *groEL* under T7 control is transformed into *E.coli* carrying a lambda prophage. The prophage's lysis genes were replaced with Lyz (endolysin), lydA (holin) and lydB (antiholin) from the lysis cassette of P1 phage by PCR and recombination. A. The lysis curve of P1 lysis cassette in this context (square) The lysis curve of *Lyz, lydA* and *lydB* the presence of overexpressed GroEL from a pRE plasmid (circle). B. To rule out GroEL interacting with *Lyz*, The same prophage was integrated into *E.coli* except *Lyz* was replaced with the lambda endolysin R.

DISCUSSION

The first step in studying S *in vitro* was to find a detergent that kept S soluble. Seventeen different detergents were tried before the zwitterionic detergent Empigen BB was discovered. The best detergent for extraction of S from the inner membrane of *E.coli* was Empigen BB. Not only that but S stayed soluble in Empigen BB for several months and also showed 54% alpha helical content by circular dichroism. This was enough helices to form three transmembrane domains, The three transmembrane domains of S was Corroborated by *in vivo* experiments (Grundling, Blasi, and Young, 2000b). This indicated that Empigen BB was able to bind and maintain S's three transmembrane domains similarly to its native S *in vivo*. The same detergent was used to purify all the S mutants with the same exact results in terms of extraction and solubility.

The second step was to look at the secondary structure of S and S mutants to determine if secondary structure was affected by different mutations. S and all S mutants showed the exact same secondary structure. No loss in helical content could be detected for S_{A52V} (which fails to trigger *in vivo*), S_{A55T} (a temperature sensitive allele), S_{A52G} (very early triggering). This showed that the loss or gain of function of each S mutant is not because of a change in its secondary structure.

The third step was to optimize the liposome assay in order to study the activity of S. Two major strides were made. First a change in lipid content, from 100% non ionic lipid

vesicles to a 70 non ionic and 30 anionic lipid content. The new lipid composition mimiced the inner membrane of *E.coli* and not surprisingly, increased the efficiency of dye release by S. If one considered the fate of S in this experiment, where a membrane protein was coming out of a chaotrope and being placed into a hydrophilic environment, then as soon as S was released into a solution it must either go into a liposome or form some type of precipitant. Since, S in the chaotrope was constantly slightly folding and unfolding in solution, one can propose that perhaps only the S that was able to quickly fold correctly and insert into the liposome once placed into the hydrophilic solution would form lesions and release dye. The negative charge on the liposomes could have enabled S to use its highly positive charged tail to bind to the liposome and gave the protein either extra time to fold and insert into the liposomes. Perhaps tail binding to the liposome actually helps in folding the protein into the right conformation. The second major stride was the discovery that GroEL bound to, protected and delivered S to liposomes. It had already been shown that excess amounts of GroEL had the same results for LacY (Bochkareva *et al.*, 1996), and that the c-terminus of GroEL promoted binding to lipid layers. The results presented here showed that GroEL could solubilize quantitative amounts of S that can be used for *in vitro* assays, but it could also deliver functional S to liposomes. The delivery of S by GroEL to liposomes gave a much higher fluorescence signal than S diluted out of a chaotrope. This

could be because GroEL binds to liposomes, thus providing both a hydrophobic environment for S until it can associate with the liposomes and a deliver mechanism. Whereas coming out of a chaotrope, S was diluted into a hydrophilic environment in which it quickly associated with liposomes or aggregates. GroEL solubilization of S provides a method to reconstitute and study S in a system that was sensitive to detergents, which has hindered the characterization of numerous membrane proteins.

Furthermore, if GroEL can solubilize membrane proteins *in vitro*, perhaps GroEL plays a similar role in solubility and/or translocation of membrane proteins *in vivo*, where it was still unclear how many post-translated membrane proteins were translocated to the inner membrane in *E.coli*, including the lambda membrane protein S. The *groE* gene was discovered as a requirement for lambda phage virion head assembly (Georgopoulos *et al.*, 1973) and it has been shown that many phages code for their own co-chaperonin that works in combination with GroEL uses (Keppel, Rychner, and Georgopoulos, 2002). In light of the results already discussed, it was interesting to test if GroEL played a role in translocation of holins from different phages. The simplest experiment was to overproduce GroEL during the late gene expression of different phages and look at the lysis timing, which was solely determined by the holin. The results varied, where overproduction of

GroEL delayed lysis for 5 minutes in the case of the lambda holin, or speeded up the lysis timing by up to forty minutes in the case of the P1 holin, lydA. These results showed that GroEL did play role a in holin activity, what role was still unclear and requireed further study. An initial study to look at whether GroEL was associated with the holin during the lytic cycle was accomplished by using immuno-pulldowns of GroEL and looking at the proteins that were associated with GroEL. A clear S band can be seen around 40 minutes after induction when GroEL was immunoprecipated. Indicating that perhaps GroEL does associate with S *in vivo*. Controls using GroEL trap, which binds but does not release substrates are needed to show that the interaction occurs prior to the lysis of the cells. Furthermore, pulse chase experiments using GroEL depletion strains can be used to monitor S accumulation in the inner-membrane. This would determine if GroEL plays a role in translocation of S to the inner membrane of *E.coli*.

CHAPTER III

FUNCTIONAL BACTERIORHODOPSIN IS EFFICIENTLY SOLUBILIZED

AND DELIVERED TO MEMBRANES BY THE CHAPERIONIN GroEL *

Soluble complexes between the tetradecameric chaperonin GroEL and integral membrane proteins can be efficiently formed by detergent dialysis. For example, $GroEL_{14}$ was found to bind a limit of two molecules of bacteriorhodopsin (BR). Single-molecule image analysis indicates that the BR molecules bind in one of the chaperonin cavities. The GroEL-solubilized BR molecules were rapidly ejected from the chaperonin complexes upon the addition of ATP or AMP-PNP, but not AMP, indicating that conformational changes induced by nucleotide binding eliminate a binding site for the hydrophobic transmembrane domains. BR retains its native conformation in the GroEL complexes, as judged by the spectral characteristics of the bound retinal. Moreover, the chaperonin-solubilized BR could be transferred efficiently to liposomes and used to effect a light-driven proton gradient, indicating that both native conformation and vectorial insertion were accomplished. These results suggest new approaches to the study of purified integral membrane proteins in their natural

membrane environment and raise the prospect that GroEL may have a role in the integration of proteins into the cytoplasmic membrane *in vivo*.

INTRODUCTION

With the exception of the proteins of the outer membranes of bacteria and related organelles, the transmembrane domains (TMDs) of integral membrane proteins are universally   a-helical and hydrophobic. Studying the structure and function of such proteins is complicated by their insolubility in the absence of detergent. Moreover, the function of these proteins is notoriously difficult to study because of the difficulty in integrating them into defined, pre-formed bilayers *in vitro*. An alternative to detergent-based solubilization was reported by (Bochkareva *et al.*, 1996), who found that a fraction of labeled LacY made in crude extract protein synthesis system could be kept from precipitating in the absence of detergent if the synthesis was done in the presence of excess GroEL. Moreover, the solubilized LacY co-migrated with GroEL and a fraction of it could be delivered to inverted membrane vesicles in native form, as assessed by cysteine-accessibility and partial proteolysis studies. The use of *in vitro* translated, labeled LacY restricted the stoichiometry to about one LacY per 25,000 added $GroEL_{14}$ molecules, and less than 10% of the labeled LacY was finally inserted into the INV, in the presence of ATP and GroES. Nevertheless, we considered these results might indicate that the chaperonin GroEL, with its hydrophobic central cavity, could be a vehicle for a robust system for delivery of purified membrane proteins to artificial membranes.

Bacteriorhodopsin (BR), represents an ideal system for testing this notion because it is a well-characterized integral membrane protein with seven a- helical TMDs and it is a light-driven proton pump that will energize membranes if delivered in its native form and in vectorial fashion. Here we report experiments exploring the capacity of the GroEL chaperonin to solubilize BR, in the absence of detergent and to deliver it in functional form to artificial membranes. The utility of GroEL for the efficient, detergent-independent delivery of proteins to membranes *in vitro* in general is discussed, as well as potential applications to structural studies of membrane proteins.

MATERIALS AND METHODS

Detergents were used at 1% final concentration. SDS-PAGE and immunoblotting have been described. Antibodies against GroEL were obtained from StressGen. Polyclonal chicken antibodies against BR were produced by Aves, Inc. against purified BR. Modifications to standard immunoblotting and immunoprecipitation protocols necessary to use the chicken antibodies were done according to manufacturer's specifications. For the immunoprecipitation experiment, primary antibodies were added to the sample at 1:2000 dilution and mixed on a roller drum at room temperature, either for 1 hour or overnight, as appropriate. Metal-decorated Magnabind secondary antibodies (Pierce) were added to the sample according to the manufacturer's instructions, and again incubated overnight on a rollerdrum at room temperature.

For each precipitation, the Magnabind antibodies and complexes were pulled to the side using a magnet-lined tube rack and washed twice. The magnetically separated pellets were analyzed by SDS-PAGE and immunoblotting.

Protein preparations

GroEL was either obtained from Stressgene (San Diego) or from an over-expression system, as described in Kamireddi et al, 1997. Results with the two preparations were identical. BR was purified from *H. halobium* as described by Oesterhelt and Stoeckenius (Oesterhelt and Stoeckenius, 1971;Oesterhelt and Stoeckenius, 1971) and delipidated as described by Huang et al.(Huang, Bayley, and Khorana, 1980;Huang, Bayley, and Khorana, 1980). The S^{21} holin was purified as described for the lambda holins, except that the detergent EBB was used instead of octyl-glucoside (OG) (Smith, Struck, et al. 1998).

GroEL solubilization of BR

GroEL at various final concentrations was added to purified BR solubilized in 1% OG, 20 mM BES, pH 7.0, 150 mM NaCl. A 1 ml sample of the mixture was placed in a dialysis bag and dialyzed overnight against the same buffer lacking detergent and in the presence of Calbiosorb Bio-Beads. Dialysis was continued until there was quantitative precipitation in a control sample containing the subject protein and the

same concentrations of BSA instead of GroEL. Visible adsorption spectra were

obtained from both the detergent-solubilized and GroEL-solubilized samples of BR,

using a Beckman-Coulter DU530 spectrophotometer. For the purpose of visualization of

the precipitate, the experiments reported in (Fig. 3-1) contained 1mg/ml BR and 20

mg/ml GroEL or BSA. In these experiments, 99.9% of the detergent was removed, as

judged by parallel experiments in which the loss of ^{3}H-labeled OG from the dialysis bag

was monitored. In 1 day of dialysis under these conditions, more than 99.8% of the

label was lost from the bag; after 2 days, less than 0.1% of the original label remained

(i.e., final OG levels were less than 0.001%). For other detergents, we assessed the

residual detergent after dialysis using a liposome-based fluorescence-release assay.

Samples were added to calcein-loaded liposomes and the dye released by liposome

destabilization monitored as fluorescence; for example, EBB as low as 0.1% caused dye

release. Dialysis under the conditions described removed the detergents to less than

10% of the critical micellar concentration.
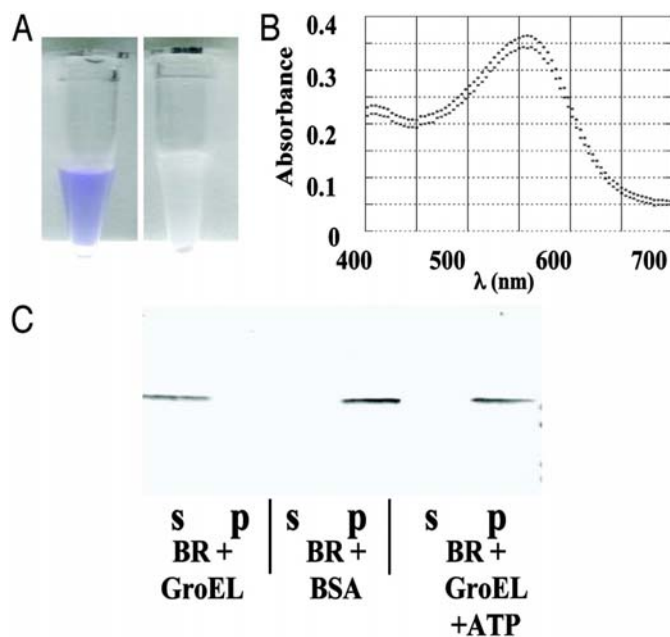
Figure 3-1. Solubilization of BR by GroEL.

A. Visualization of BR solubilized by GroEL. BR at 1 mg/ml in 1% OG was dialyzed in the presence of GroEL or BSA at 20 mg/ml, as described in Methods. After dialysis, the samples were centrifuged at maximum speed for 1 min in a microcentrifuge. BR retained its normal color with GroEL, as can be seen from the spectrum. and no precipitate was observed, whereas with BSA a colorless precipitate was formed (right).

B. BR solubilized by GroEL is native. Absorption spectra of BR (0.5 mg/ml) solubilized in OG (1%; o) or in GroEL (8.35 mg/ml; χ).

C. BR is quantitatively solubilized in an ATP-sensitive fashion by GroEL. BR at 10 mg/ml in 1% OG was dialyzed with 200 mg/ml GroEL or 200 mg/ml BSA as described. Soluble (s) and insoluble pellet (p) fractions obtained by centrifugation as described in **A** were analyzed by SDS-PAGE and immunoblotting with anti-BR antibodies. The right-most two lanes represent a BR sample dialyzed with GroEL but with 5 mM ATP added to the dialysis buffer.

To test the effects of nucleotides on the solubilization process, ATP, ADP or AMP (at 5mM) were added to the dialysis solution. To assess the effects of nucleotides on the stability of the complexes between the membrane proteins and GroEL, the same nucleotides or AMP-PNP were added directly, at 5 mM final concentration, to the solubilized protein. Precipitation, when observed, was complete within seconds for ATP, as compared to several hours for AMP-PNP and several days for ADP.

Analytical gel filtration chromatography

Gel filtration analyses were performed on an AKTA (Amersham Pharmacia) workstation. Samples were filtered through a 0.22-micrometer-pore-size sterilization filter, and concentrated to 1 ml by centrifugation in an Amicon Centriplus at 3,100 rpm, according to the manufacturer's instructions. The resulting 1-ml samples were mixed with Pharmacia high- and low-molecular-weight gel filtration calibration kit markers, chromatographed on a 24 ml Superose 6 10/300 GL column, and collected in 24 1-ml fractions, all according to the manufacturer's instructions. All fractions were analyzed by immunoblotting. As controls, samples and markers were also resolved separately on the column.

Assay of BR function delivered to liposomes by GroEL complexes

Liposomes were prepared as described previously. GroEL was loaded with BR (20 mg BR and 400 mg GroEL; approximately 1.6:1 BR to GroEL) by dialysis. The BR-GroEL solution, or GroEL alone, was added to 80 ml of the liposomes, in a volume of 1.2 ml of TBS (10 mM Tris-HCl, pH7.6; 150 mM NaCl), and stirred for 24hrs in the

dark at room temperature. Fifty microliters of TBS saturated with 9-AA was added to the liposome solution, and the mixtures were brought to 2 ml with TBS. Fluorescence was followed in an Aminco-Bowman Series 2 spectrofluorometer.

To assess the efficiency of this delivery, a BR-GroEL-liposome mixture in a total volume of 40 ml were placed on ice, adjusted to 50% sucrose by the addition of 73% sucrose (wt/vol in TBS), and overlaid with 150 µl 40% sucrose and then 300 µl of 25% sucrose in a 1µl Ultraclear centrifuge tube (Beckman). After centrifugation at 269,000×g for 3hr at 4C in a Beckman TLA-100.3 table top ultracentrifuge rotor, seven 100 µl fractions were drawn from the air-fluid interface. Fractions 1-3 and 5-7 were pooled as the top (floated) and bottom fractions, respectively, concentrated to 30 µl using a centrifugal concentrator (Millipore Microcon YM-3), according to the manufacturer's instructions, and analyzed for BR content by A_{555}, the absorption maximum for bound retinal. The three top fractions contained 100% of the lipid, as judged by parallel flotation experiments using liposomes where 0.5% of the phosphatidylethanolamine was replaced with the fluorescent analog phosophatidylethanoline-N-lissamine rhodamine B (Avanti).

Electron microscopy and single particle analysis

GroEL (0.1 mg/ml) or GroEL loaded with BR were negatively stained using an aqueoussolution of uranyl acetate (1% w/v, p.H4.3) (Hoppert and Holzenburg, 1998). Specimens were observed in a JEOL 1200 EX transmission electron microscope operated at an acceleration voltage of 100 kV. Images were recorded at a calibrated magnification of 40,800x. Selected micrographs were digitized using the Leafscan 45 microdensitometer at a scan step corresponding to 0.25 nm/pixel at the specimen level. Single particle analysis was carried out using the EMAN software package (Ludtke, Baldwin, et al. 1999). Approximately 1000 particles of GroEL with and without BR were selected for image processing. After bandpass filtering and translational alignment of the particles, a first structure was calculated in C7. Iterative refinement of the three-dimensional model was carried out by comparing raw particles with reprojections of the previous three-dimensional structure. With each round of refinement new class averages were calculated and back-projected into predefined orientations. Fourier shell correlation was used to monitor the convergence of the reconstructions and the final resolution was 2.8 to 3.0 nm. Surface rendering of the final three-dimensional structures was carried out using the VIS5D software package (see http://vis5d.sourceforge.net/). The mass threshold for GroEL and GroEL-BR was set to 840kDa.

RESULTS

Solubilization of BR

Purified BR at 1 mg/ml solubilized in 1% OG was placed in a dialysis bag with 20mg/ml of either GroEL or BSA (as a control), and subjected to dialysis. After 12hrs, the control chamber showed visible precipitate (Fig. 3-1A), while the GroEL chamber remained clear and with the characteristic purple color of native BR; moreover, the absorption spectra from 350 nm to 700nm, normalized for the amount of protein (A_{280}) were identical for the input and solubilized BR, demonstrating that no BR was denatured during the dialysis (Fig. 3-1B). SDS-PAGE and Western blotting showed that the BR was quantitatively solubilized in the presence of GroEL(Fig. 3-1C). The BR remained soluble in the presence of GroEL for at least 90 days in the cold.

Stoichiometry of BR solubilization by GroEL

By titrating in increasing amounts of BR into these solubilization mixtures and measuring the soluble component by densitometric analysis of Coomassie-blue stained protein gels, the capacity for solubilization was determined to be 2 molecules per $GroEL_{14}$, or 52 kDa (Fig.3-2). Interestingly, BR solubilized in EBB, where it is stable for less than 2 weeks, is relatively poorly retained in solution in the presence of GroEL, with about half of the BR escaping precipitation during dialysis (Table 3-1). This
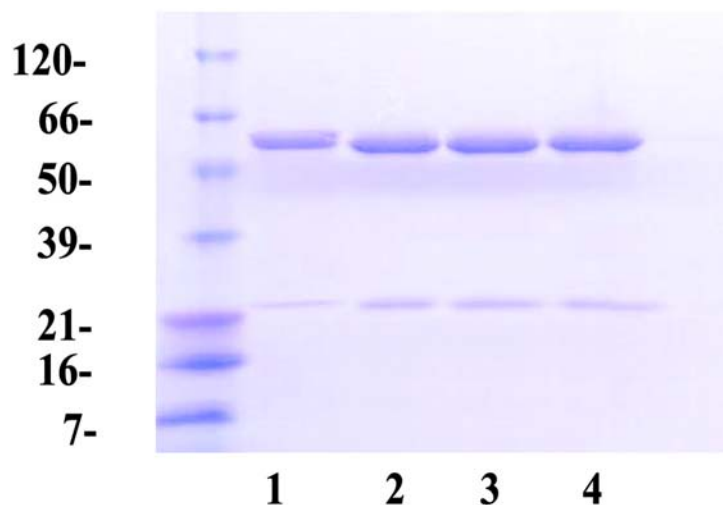
Figure 3-2. Capacity for BR is 2 molecules per GroEL.

GroEL (1 mg) was mixed with OG-solubilized BR at various ratios of BR to tetradecamer in 1 ml final volume and then subjected to dialysis to remove detergent, as described in Methods. Insoluble material was removed by centrifugation and the soluble material analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue. Ratios of BR to $GroEL_{14}$ in lanes 1 through 4 were 1:1, 2:1, 5:1, 10:1, respectively. Protein amounts were determined by densitometry. Mass standards in kDa are shown to the left.

indicates that the GroEL-solubilization of membrane proteins is dependent on the original detergent, perhaps because the process of binding to GroEL competes with pathways leading to insoluble aggregates.

GroEL forms a complex with BR

To investigate the molecular basis of the GroEL-mediated solubilization, both detergent and GroEL-solubilized forms of BR were subjected to gel filtration chromatography, in parallel with GroEL itself. All of the solubilized BR migrates with GroEL in gel filtration experiments, without significantly affecting the apparent mass of the tetradecamer (Fig. 3-3), as would be expected from the relative sizes of BR and the chaperonin. Immunoprecipitation with GroEL antibodies quantitatively co-precipitated BR (Fig. 3-4). Reciprocal immunoprecipitation with polyclonal anti-BR antibodies also resulted in complete precipitation of GroEL (Fig. 3-4), when the input ratio of BR to GroEL was 1:1. This result suggests that under the conditions of loading by slow removal of detergent, 1 BR is bound per tetradecameric chaperonin before a second molecule is bound. We conclude that the solubilization obtained by removing detergent in the presence of GroEL results in the formation of a complex between the membrane proteins and the GroEL chaperonin. Moreover, in the case of BR, where the solubilization does not exceed the projected capacity of the GroEL cavity, BR is still freely accessible to polyclonal antibodies.
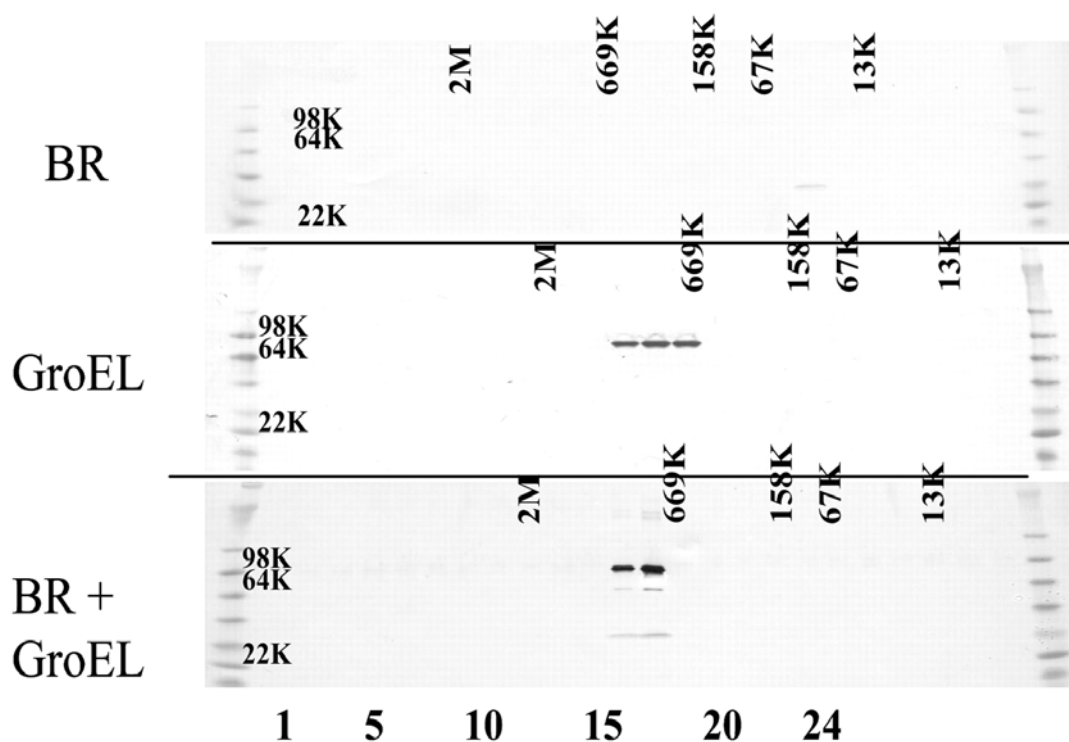
Figure 3-3. Gel filtration of GroEL-solubilized BR.

BR solubilized in 1% OG (top panel), GroEL (middle panel) and BR solubilized by GroEL (bottom panel) were analyzed by gel filtration on a Superose 6 10/300 GL column. All 24 fractions were collected and analyzed by SDS-PAGE and immunoblotting with anti-BR (top panel), anti-GroEL (middle panel) or both antibodies (bottom panel). For these experiments, 30 mg BR and 1 mg GroEL were used (a ratio of BR to GroEL$_{14}$ of 1:1.) Positions of size standards are indicated for each chromatographic run.
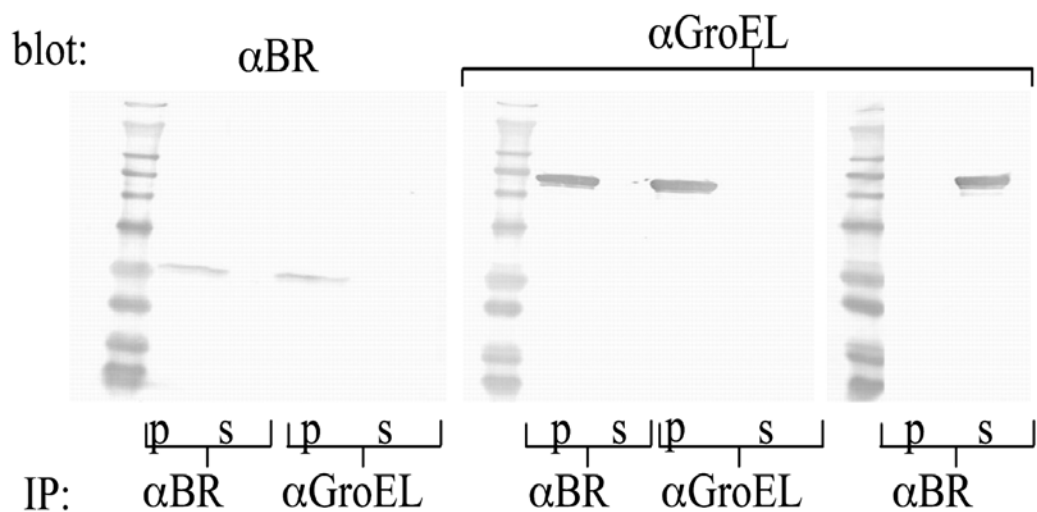
Figure 3-4. Quantitative co-immunoprecipitation of GroEL and solubilized BR.

A mixture of 3 mg/ml BR and 100 mg/ml GroEL (1:1 BR-GroEL), after removal of detergent by dialysis, was subjected to immunoprecipitation and analysis by SDS-PAGE and immunoblotting with either anti-BR (left panel) or anti-GroEL (middle and right panels).   Immunoprecipitations (IP) were done with the antibodies indicated below, with p = immunoprecipitated fraction and s = supernatant fraction. Right panel: a control sample of GroEL subjected to immunoprecipitation with anti-BR.

Effects of nucleotides

ATP binding by GroEL has been shown to cause a conformational change resulting in an expansion of the opening to the cavity of the chaperonin, causing a reduced affinity of the chaperonin complex for unfolded protein substrates. This change does not require ATP hydrolysis, is much less dramatic with ADP and does not occur with AMP (Roseman *et al.*, 2001). To test whether the BR-GroEL solubilization complex was sensitive to nucleotide binding, 5 mM ATP was added to the dialysis buffer in the BR-GroEL solubilization experiment; under these conditions, a precipitate containing all of the BR formed with the same kinetics as in the control containing BSA (Fig. 3-1B; Table 3-1). Moreover, ATP added to pre-formed BR-GroEL complexes caused immediate precipitation of BR (not shown). We conclude that an ATP-sensitive conformation of GroEL is required for the solubilization, suggesting that the apical domain of the chaperonin is involved and that the solubilization complex may be analogous to the complex formed upon initial binding of unfolded polypeptides to GroEL. In contrast, ADP caused only a very slow release of BR, and AMP was without effect. AMP-PNP also caused release of BR from the GroEL complex, indicating that the release process does not require ATP hydrolysis (Table 3-1); however, with AMP-PNP the formation of the visible precipitate required 1-2 hours, presumably reflecting the lower affinity of the ATP binding sites on GroEL for the analog.

Structure of BR-GroEL complex

Electron micrographs of GroEL with and without BR were subjected to single particle analysis and 3-D reconstruction (Fig. 3-5). Applying the Fourier shell correlation criterion suggests that the two structures show consistent features to at least 2.5 nm resolution and that the structure of BR-GroEL is similar to empty GroEL as far as GroEL-specific densities are concerned. The readily discernible difference is that both the BR-GroEL and BR_2-GroEL complexes display extra mass in only one of the two cavities of GroEL. This finding suggests that BR binds to GroEL in a way similar to what is observed with denatured soluble proteins (Rye *et al.*, 1999).

Solubilization of other integral membrane proteins

The GroEL-dependent solubilization is not limited to BR. In addition, the *E. coli* maltose-aspartate chemoreceptor Tar, with four TMDs per dimer, and the maltose permease complex, consisting of the $MalGFK_2$ heterotetramer, with 14 TMDs, also remained soluble upon removal of detergent by dialysis in the presence of GroEL (Table 3-1). Under the same conditions, detergent removal in the presence of BSA resulted in quantitative precipitation of the proteins. Although titrations were not performed on Tar and $MalGFK_2$, both were solubilized at approximately 1:1 with the $GroEL_{14}$ complex. In the membrane, Tar is a dimer of about 120 kDa, and the Mal proteins constitute a transport complex of over 170 kDa. These masses would seem to be significantly in

excess of the estimated capacity of the Luminal cavity of the GroEL complex, which has been reported to be about 60 kDa (Ewalt *et al.*, 1997), although the encapsulation of an 86 kDa heterodimer has been reported (Song *et al.*, 2003). However, unlike BR, the hydrophobic domains of these complexes constitute only a fraction of the total mass, and thus it is possible in these cases that only the TMDs are bound in the lumen. One integral membrane protein, *E. coli* lactose permease, LacY, with 12 TMDs, exhibited transient solubilization, in that, during dialysis to remove the detergent dodecylmaltoside (DM), complete precipitation was observed in the control experiment with BSA more than two days before precipitation was detectable with GroEL present. Another protein, *E. coli* diacylglycerolkinase, DAGK, showed no GroEL-dependent solubilization when the original detergent was EBB. However, DAGK in DM mirrored LacY in showing transient solubilization, again with precipitation in the BSA control more than two days before precipitation in the presence of GroEL. Finally, one of the proteins tested showed no detectable solubilization under the same conditions: the type II holin from the lambdoid phage 21, $S^{21}68$, with two TMDs (Table 3-1).

The BR-GroEL complex can deliver functional BR to pre-formed liposomes

Throughout the solubilization procedure, the BR-GroEL complexes retained the characteristic purple color of native BR. To determine whether these complexes formed from purified proteins could support integration of the functional BR into the bilayer,

Table 3-1. Solubilization of membrane proteins by GroEL.

Membrane protein[1]	Input ratio[2]	% Soluble[3]	#[4]
MalGFK2 (EBB)	1:1[5]	100	1
Tar (EBB)	10:1	100	1
	1:1	100	1
BR (EBB)	1:1	50	2
BR (OG)	10:1	100	2
	1:1	100	>3
	1:2	100	>10
	1:3	80	2
	1:10	<40	2
	1:20	<30	1
+ATP (5mM)	1:2	0	1
+ADP (5mM)	1:2	NA	1
+AMP (5mM)	1:2	100	1
+AMPPNP (5mM)	1:2	0	1
$S^{21}68$ (EBB)	100:1	0	2
	10:1	0	2
	1:1	0	4
	1:2	0	2
	1:10	0	3
	1:100	0	3
	1:300	0	2
DAGK (DM)	1:1	20[3]	1
	1:10	~1[3]	1
DAGK (EBB)	1:1	0	1
	1:10	0	1
LacY (DM)	1:1	20[3]	1
	1:10	~1[3]	1

[1]Protein samples purified in indicated detergent as specified in Experimental Procedures. GroEL present at 100 mg/ml. Detergents, indicated in parenthesis, were present at concentrations indicated in Methods.

[2]Ratio of $GroEL_{14}$ molecules to solute protein molecules.

[3]Estimated from intensity of immunoblot bands. See Fig. 1B. For LacY and DAGK, solubilization was temporary; solute began precipitating approximately 3 days after the control precipitated.

[4] Number of experiments

liposomes labeled with 9-AA, a fluorescent dye sensitive to the electrochemical proton gradient, were mixed with either GroEL-solubilized BR, or GroEL alone, and incubated for 24 hours. The function of BR was assessed by the ability to generate a light-dependent proton gradient, measurable as quenching of 9-AA fluorescence. In the liposomes exposed to the BR-GroEL complexes, excitation of BR resulted in quenching of 9-AA fluorescence, whereas no such quenching was obtained with the GroEL-only control (Fig. 3-5). Addition of 0.5 mM dinitrophenol immediately abolished the quenching, even if excitation of BR was continued (not shown). To estimate the efficiency of delivery, a parallel experiment was done in which the liposomes were separated from the GroEL mixture by flotation. The floated fraction, containing 100% of the lipid, also contained approximately 29% of the BR (as judged by A_{555}) and less than 10% of the GroEL, as judged by quantitative immunoblotting (not shown). Approximately 59% of the BR remained with the GroEL fractions; thus about 12% of the A_{555} was lost, presumably due to loss of retinal from the BR. Moreover, washing the liposomes with 0.5M NaCl had no effect on the amount of BR bound (not shown), confirming that the BR molecules are integrated within the bilayer. Although no attempt was made to optimize this delivery system, these results clearly demonstrate that the BR-GroEL complexes can support efficient vectorial insertion of functional BR into a preformed bilayer.
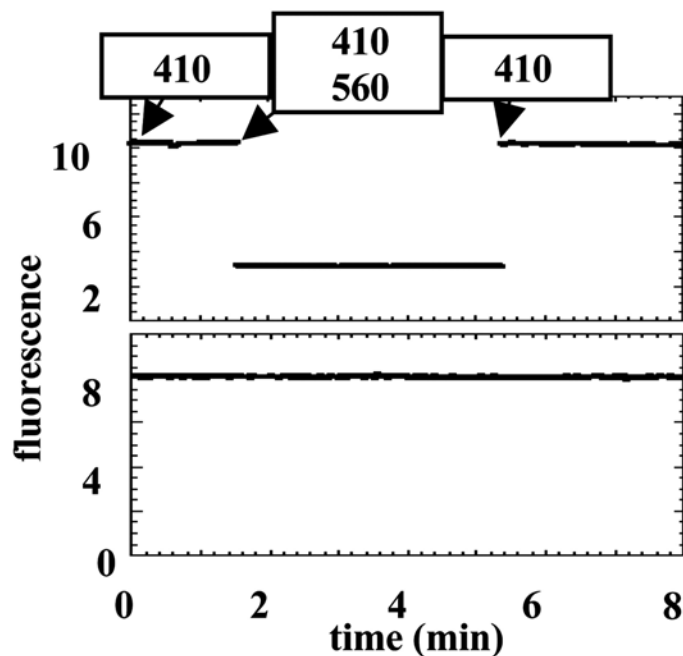
Figure 3-5. BR delivered by GroEL complexes can form a light-dependent proton gradient.

Large unilamellar liposomes prepared from 70:30 acidic:neutral phospholipids and loaded in buffer containing 9-AA were incubated with BR-GroEL complexes (formed at 1.6 BR per GroEL$_{14}$; top panel) or with GroEL alone (bottom panel). After 24 h, the liposomes were illuminated with 410 nm light to excite the 9-AA. Between 2 and 5 min after onset of illumination, the liposomes were additionally illuminated with 560nm light, to power the BR proton pump. 9-AA fluorescence was monitored throughout at 430 nm. There is a 30-40 second time lapse when excitation wavelengths are changed, due to manual exchange of filters. When the experiment was repeated without liposomes, no change in the 9AA fluorescence was observed upon illumination with 560 nm light (not shown).

DISCUSSION

Quantitative detergent-free solubilization of BR by the GroEL **chaperonin**

The work of Bochkareva (Bochkareva *et al.*, 1996) showed that about 30% of the lactose permease (LacY) synthesized *in vitro* in the presence of a great molar excess of GroEL could be retained in a soluble state in the absence of detergent. Here we extend this seminal finding by reporting that high microgram to milligram quantities of a purified, native integral membrane protein, BR, with 7 a-helical TMDs, can be efficiently transferred from a detergent-solubilized state to a detergent-free complex with the chaperonin $GroEL_{14}$. Moreover, this solubilization occurs efficiently and completely even in a 2:1 molar excess of BR over the tetradecameric chaperonin, and at up to low milligram concentrations of BR. As noted originally (Bochkareva *et al.*, 1996) unlike soluble proteins which generally must be denatured to expose the hydrophobic surfaces necessary for binding in the $GroEL_{14}$ cavity, integral membrane proteins could be bound in their native state. BR contains one molecule of the chromophore retinal, conferring a characteristic purple color on solutions of the purified native protein; this color is unaffected by the removal of detergent and the formation of the BR-GroEL complexes, indicating that the native conformation of BR is not compromised. By the same criterion, the BR-GroEL complex is stable for weeks in the cold. Irrespective of the molecular details of this phenomenon, it is likely to be extremely useful for the study of BR, a paradigm energy-transducing protein, and, by extension, for the study of the structure and function of integral membrane proteins in general. The ease by which

GroEL can be purified in quantity(Kamireddi, Eisenstein, and Reddy, 1997) and its commercial availability is worth noting in this regard.

Can GroEL be a general vector for integral membrane proteins?

Our preliminary survey, using only a narrow set of conditions, found that while several other integral membrane proteins were solubilized if subjected to removal of detergent in the presence of GroEL, others were not (Table 3-1).   There is no discernible pattern, either in terms of bulky hydrophilic domains, number of TMDs or charge distribution within aqueous domains (not shown), for the membrane proteins that are not solubilized by this technique.   The GroEL-mediated retardation of the precipitation of LacY during detergent removal suggests that for any cytoplasmic membrane protein, GroEL-mediated solubilization may reflect a competition,   as detergent is removed, between unproductive, irreversible aggregation and a reversible partition into GroEL complexes. According to this view, the GroEL-solubilized state is kinetically trapped, presumably by the slow off-rate from the cavity of GroEL. BR is more efficiently transferred to GroEL from OG than from EBB, mirroring the increased stability of BR in the former detergent. This suggests that OG stabilizes BR in a conformation more compatible with transfer to GroEL than does the zwitterionic

detergent. Some LacY associates stably with $GroEL_{14}$ if DM-solubilized LacY is subjected to very rapid removal of detergent. Again, this suggests that DM preserves LacY in a conformation that more rapidly associates with GroEL, relative to the formation of precipitating aggregates. Whether or not GroEL can be made to be a general vector for solubilization and delivery of membrane proteins is still uncertain, but we think it likely. If the binding to GroEL is a process that competes with hydrophobic aggregation as detergent is removed by dialysis, then systematically varying the detergent and the rate of dialysis may point to conditions where any membrane protein can be efficiently partitioned into GroEL.

Structure and assembly of the BR-GroEL complex

Titration of BR into the GroEL chaperonin revealed that a maximum of 2 BR molecules could be bound. When the BR-GroEL complexes were examined by electron microscopy, two-dimensional difference maps of GroEL and BR-GroEL revealed that the extra density in the BR-bound structures was restricted to the chamber formed by one heptamer, irrespective of the number of BR molecules bound. The molecular basis for this may be that the heptamer without the increased mass appears to have undergone a conformational change leading to a narrowing of the apical opening (Fig. 3-5). Similar changes have been observed for the empty chamber in the GroES-ATP mediated folding

cycle observed for the GroEL chaperonin (Rye *et al.*, 1999). The binding of the second molecule of BR into the BR-containing chamber may be facilitated by interactions between the incoming BR and the BR bound near the apical domain. Finally, it is possible that the binding of BR and other membrane proteins may generate conformational asymmetry in the tetradecamer, and thus high-resolution structures of membrane proteins may be obtained by loading them first into GroEL. Efforts to assess whether this is practical are now in progress.

Delivery of BR to membranes from the BR-GroEL complex

Bochkareva (Bochkareva *et al.*, 1996) showed that a fraction of in-vitro synthesized LacY bound to GroEL could be transferred to inverted membrane vesicles, but not to right-side out vesicles; this transfer was most efficient in the presence of ATP and GroES. Moreover, at least some of the permease molecules attained a native conformation in the membranes, as judged by protection of a sensitive cysteine thiol by a substrate analog. These studies did not allow determination of whether it was essential that the LacY cargo be co-translationally bound to GroEL or whether the cellular machinery for the protein secretion or membrane integration and secretion was required for the insertion into the membrane. Similar findings were made by Meryandini and Drews (Meryandini and Drews, 1996)using an *in vitro* translation system and membranes from *Rhodobacter capsulatus*. Here we have shown that purified GroEL

can not only efficiently bind native, purified BR but also deliver it in native form to artificial membranes, in the absence of ATP or GroES. In addition, we were able to demonstrate that the BR integrated into the liposomes was functional, as judged by its ability to conduct light-dependent proton pumping (Fig. 3-6).   Thus, for BR, it is not necessary for GroEL to take up the membrane protein co-translationally, and no proteinaceous machinery or added ATP is required for vectorial insertion of BR, in its functional, native state, into a bilayer from the GroEL complexes.

About 30% of the BR was delivered to the liposomes under the one set of conditions used. It seems likely that with systematic analysis of the stoichiometry, kinetics or efficiency of the integration step, quantitative transfer to the bilayer can be obtained. With another integral membrane protein, the lambda holin, we have found up to 50% is found associated with the liposomes after 1 hour of incubation with loaded chaperonin complexes (J. Deaton, C. Savva, J. Sun, A. Holzenburg and R. Young, manuscript in preparation). Like the selection of detergent and dialysis conditions for the formation of complexes with GroEL, the efficiency of delivery to artificial membranes is likely to require optimization of conditions, including the ionic and osmotic characteristics of the solution, the lipid content of the liposomes, and the concentration and stoichiometry of the chaperonin complexes and the liposomes. The fluorescence of the 9-AA is efficiently quenched when the liposomes inserted with BR

are illuminated with visible light (Fig. 3-6), indicating that BR molecules are inserted vectorially in the bilayer. Our results appear to be inconsistent with one of the findings of (Bochkareva *et al.*, 1996), in that they observed that LacY complexes with GroEL supported membrane insertion only with inverted membrane vesicles, not with right-side out vesicles derived from whole cells. Of course, it is possible that this reflects some intrinsic difference between LacY and BR. However, it seems more likely that their observation might reflect the vanishingly small amount of labeled LacY in the incubation mixtures. Perhaps the inner leaflet of INV contains high affinity sites for GroEL complexes (i.e., the SecY-SecA translocon), as suggested by the same authors in a subsequent study (Bochkareva *et al.*, 1996). This could account for the ATP and GroES dependency as well. In any case, the suggestion by these authors that GroEL may have a biological role in the insertion of integral membrane proteins into the bilayer is supported by the robust character of the GroEL-dependent solubilization of BR and other membrane proteins, and the ability of GroEL to deliver BR to pre-formed bilayers.

CHAPTER IV

SOLUBILIZATION AND DELIVERY BY GroEL OF MEGADALTON

COMPLEXES OF THE LAMBDA HOLIN*

GroEL can solubilize membrane proteins by binding them in its hydrophobic cavity when detergent is removed by dialysis. The best-studied example is bacteriorhodopsin, which can bind in the GroEL chaperonin at 2 molecules per tetradecamer. Applying this approach to the holin and antiholin proteins of phage lambda, we find that both proteins are solubilized by GroEL, in an ATP-sensitive mode, but to a vastly different extent. The antiholin product, S107, saturates the chaperonin at 6 molecules per tetradecameric complex, whereas the holin, S105, which is missing the two N-terminal residues of S107, is solubilized at up to 350 holin molecules per GroEL, or approximately 4 MDa of protein per 0.8 MDa tetradecamer. Gel filtration chromatography and immunoprecipitation experiments confirmed the existence of complexes of the predicted masses for both S105 and S107 solubilization. For S105, negatively stained electron microscopic images show structures consistent with protein

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shells of the holin assembled around the chaperonin tetradecamer. Importantly, S105 can be delivered rapidly and efficiently to artificial liposomes from these complexes. In these delivery experiments, the holin exhibits efficient membrane-permeabilizing activity. The S107 antiholin can block formation of the hyper-solubilization complexes, suggesting that their formation is related to an oligomerization step intrinsic to holin function.

INTRODUCTION

With the exception of the proteins of the outer membranes of bacteria and related organelles, the transmembrane domains (TMDs) of integral membrane proteins are universally α-helical and hydrophobic. Studying the structure and function of such proteins is complicated by their insolubility in the absence of detergent. Moreover, the function of these proteins is notoriously difficult to study because of the difficulty in integrating them into defined, pre-formed bilayers *in vitro*. Recently we have found that integral membrane proteins can be kept soluble without detergent by binding to the GroEL tetradecameric chaperone (Deaton *et al.*, 2004a). Definitive experiments were performed with purified bacteriorhodopsin (BR), an integral membrane protein with seven transmembrane domains. After dialysis of detergent-solubilized BR in the presence of GroEL, it was found that BR-GroEL complexes were formed containing two

molecules of BR at saturation. The complexes were sensitive to the presence of ATP, but not AMP. Remarkably, BR retained native conformation in the complexes, which could be used to deliver BR to liposomes efficiently, vectorially, and in functional form.

Our original motive to develop methods for the detergent-free solubilization and delivery of membrane proteins derived from our interest in the function of the bacteriophage lambda holin protein. Holins are integral membrane proteins that cause a temporally-scheduled permeabilization of the membrane during host cell lysis (Wang, Smith, and Young, 2000). The holin protein, S105, and the antiholin, S107, which binds to and inhibits S105, are both products of the *S* gene, differing only by the N-terminal Met-Lys extension at the N-terminus of S107. Hole-formation is thought to depend on oligomerization of the holin, and some mutants defective in lysis, such as S105a52v, appear to be blocked at the dimer stage (Grundling, Blasi, and Young, 2000c).

In view of the results with BR, we considered that GroEL might form complexes with substantial amounts of purified S105 and S107 and that such complexes might be used to insert the *S* gene products into artificial membranes. Experiments to test this are reported here. The results are discussed in terms of the nature of the S-GroEL interaction, the fundamental functional properties of holin proteins and the possible role of GroEL in the insertion of membrane proteins *in vivo*.

MATERIALS AND METHODS

Calbiochem was the source for all detergents, Calbiosorb Biobeads, and ATP. Bovine serum albumin (BSA), AMP, ADP and AMP-PNP were obtained from Sigma. BES was obtained from USB. Detergents were used at 1% final concentration. When necessary, samples were concentrated using a Microcon centrifugal filter unit (Amicon), with a molecular weight cut-off of 3000, according to the manufacturer's instructions. To concentrate protein for SDS-PAGE, 100 ml samples were precipitated by addition of 2 ml of cold 95% EtOH; pellets were recovered by centrifugation for 10 min at 1,000 x g and dissolved in sample buffer. SDS-PAGE and immunoblotting have been described. The antibodies recognizing *S* gene products have been described (Chang, Nam, and Young, 1995). Antibodies against GroEL were obtained from StressGen. Polyclonal chicken antibodies against BR were produced by Aves, Inc. against purified BR. Modifications to standard immunoblotting and immunoprecipitation protocols necessary to use the chicken antibodies were done according to manufacturer's specifications. For immunoprecipitation experiments, primary antibodies were added to the sample at a dilution of 1:2000 and incubated on a roller drum for one hour at room temperature. Metal-decorated Magnabind secondary antibodies (Pierce) were added to the sample according to the manufacturer's instructions, and again incubated for one hour on a roller drum at room temperature. For each precipitation, the Magnabind antibodies and

complexes were pulled to the side using a magnet-lined tube rack and washed twice; this procedure prevents contamination of the immune complexes with proteins that spontaneously precipitate out of solution. The magnetically-separated complexes were analyzed by SDS-PAGE and immunoblotting.

Gel filtration chromatography

Gel filtration analyses were performed on an AKTA (Amersham Pharmacia) workstation. Samples were filtered through a 0.22-mm-pore-size sterilization filter, and concentrated to 1 ml. Samples were chromatographed on a Superose 6 10/300 GL column and collected in 1 ml fractions. The column was calibrated with the following molecular weight standards: polystyrene (~6 MDa); Blue Dextran 2000 (~2 MDa); thyroglobulin (670 KDa); ferritin (440 KDa); chymotrypsinogen (25 KDa), according to the manufacturer's instructions. For analytical purposes, eluted fractions were concentrated by cold ethanol precipitation and analyzed by SDS-PAGE and immunoblotting. For liposome permeabilization assays, selected fractions were concentrated and used without further manipulation.

Protein preparations

Purified GroEL was initially obtained from Stressgene (San Diego). Subsequent experiments were done with GroEL purified from an over-expression system, as described in (Kamireddi, Eisenstein, and Reddy, 1997). The two preparations behaved identically in our hands.

Oligohistidine–tagged holin and antiholin proteins were purified as described, with some modifications. Membranes from cells producing these proteins were subjected to differential solubilization in 1% EBB, 10% glycerol, 20mM BES, 0.5M NaCl, 35 mM MgCl$_2$ pH 8.0 for 2 to 18 hours at 37C with shaking. Insoluble material was removed by centrifigation at 100,000g for 45 minutes and the soluble extract was filtered through a 22 mm syringe filter (Genemate Bioexpress) and loaded onto a Hitrap Chelating HP nitrilo-triacetic acid column (1 ml) charged with CoCl$_2$ and equilibrated with 1% EBB, 20mM BES, 0.5M NaCl, 10% glycerol, pH 7.6. After loading, the column was washed with 1% EBB, 20mM BES, pH 7.6 and eluted at a flow rate of 0.5ml/minute with a pH gradient from pH 7.6 to pH 2.5, using 1% EBB, 20mM sodium acetate, 0.5M NaCl as the limit buffer. Oligohistidine-tagged proteins were eluted with a pH gradient Eluted protein fractions were neutralized with 0.1 M NaOH.

GroEL solubilization of holins

To form complexes between *S* gene products and GroEL, 800 ml of 1% EBB, 20mM BES, 0.5M NaCl, pH 7.6 was placed in a tube and 100 ml of a GroEL solution in the same buffer was added. Finally, 100 ml of S protein in the same buffer was added. At each step, the solution was mixed by pipet. For most experiments, the final concentration of GroEL was 100 mg/ml, and the concentration of S protein was adjusted to achieve the desired molar ratio to the tetradecameric chaperonin. For the purpose of

visualization of the precipitate, some experiments contained a final concentration of 2 mg/ml S105 and 1 mg/ml GroEL or BSA. For titration experiments, the final concentration of GroEL was 50 mg/ml for S105 and 75 mg/ml for S107. The 1 ml solution containing detergent-solubilized S protein and GroEL was placed into a dialysis bag and dialyzed against 500 mls of 20mM BES, 0.5M NaCl, pH 7.6, supplemented with Calbiosorb Bio-Beads, according to the manufacturer's instructions. Buffer and Bio-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 BSA. In these experiments, the efficacy of detergent removal was assessed using calcein-loaded liposomes as previously described (Smith, Struck, et al. 1998). In all cases, detergent was reduced to less than 10% of its critical micellar concentration.

To test the effects of nucleotides on the solubilization process, 5 mM ATP, ADP or AMP was added to the dialysis solution. To assess the effects of nucleotides on the stability of the complexes between the membrane proteins and GroEL, the same nucleotides or AMP-PNP were added directly, at 5 mM final concentration.

Electron microscopy of Nanogold-labeled S-GroEL complexes

Mono-NHS-Nanogold (6 nM; Nanoprobes, Yaphank, NY) was dissolved in 100 ml 10% DMSO. Labeling was initiated by adding 80 ml of S105 (2.6 mg/ml in 1% EBB, 0.5M NaCl, 20 mM BES buffer, pH.7.8) to 40 ml of Nanogold. After incubation at 4C overnight, the mixture was subjected to gel filtration through a Sephadex 75 column (1.5 cm by 25 cm) on an AKTA FPLC (Pharmacia). Fractions (1 ml) were collected and analyzed by SDS-PAGE, rendered non-reducing by omitting β-mercaptoethanol from the sample buffer. Duplicate gels were stained with Coomassie Brilliant Blue and LI-Silver (Nanoprobes) for detection of protein and nanogold, respectively. The fractions containing the conjugate were pooled and concentrated to 200 ml. The concentrated conjugates were then incubated with 75 ml of GroEL (1 mg/ml in 10mM KCl, 10mM $MgCl_2$, 10 mM Tris, pH7.6) and dialyzed as described. Control samples were prepared by adding equivalent amounts of GroEL and de-activated nanogold, incubated for 3 days in 200 mM Tris-HCl, pH 7.5.

S105-Nanogold/GroEL and control samples were prepared for electron microscopy by adding 4 ml of sample onto formvar-carbon coated copper grids (400 mesh) that had previously been rendered hydrophilic by glow-discharging. After incubation for 60 sec, the samples were blotted to remove excess solution and stained with Nanovan (Nanoprobes). Duplicate grids were prepared similarly but stained with

2% uranyl acetate and 2% ammonium molybdate. Samples were examined using a Zeiss 10C electron microscope operating at 80 kV. Micrographs were taken at a calibrated magnification of 55,400.

in vitro membrane permeabilization assays

Liposomes were made by mixing 245 µl of dioleyl-L-a-phosphatidylcholine (20 mg/ml)), 210 µl of dioleylphosphatidylglycerol (10 mg/ml) and 143 µl of cholesterol (7 mg/ml) in chloroform (Avanti Polar Lipids Inc., Alabaster, AL). The mixture was dried under a stream of nitrogen and suspended in 1 ml solution containing 0.15 M of the self-quenching fluorophore calcein (in 10 mM Tris and 150 mM NaCl; pH adjusted to approximately 7.6 with 2M NaOH). The resulting lipid suspension was extruded 40 times through a 200nm polycarbonate membrane in a Liposofast extrusion device (Avestin Inc., Ottawa, ON, Canada). The mainly unilamellar liposomes loaded with calcein were separated from unincorporated calcein by gel filtration chromatography on a G-50 Sephadex column (1.5 cm by 25 cm) equilibrated in TBS (10mM Tris pH 7.6, 150mM NaCl). Fractions at the void volume containing the liposomes were clearly visible and were collected manually.

The permeabilization of the calcein-loaded liposomes was measured using a SLM 8100 spectrofluorometer (Spectronic Instruments, Inc. Rochester, NY) with excitation and emission set at 490nm and 520nm, respectively. The reaction mixture contained 40 ml of the liposome suspension (28 mg lipid, $\sim 10^{12}$ liposomes) in a final volume of 2 ml of TBS. The assay was initiated by the addition of the indicated protein sample in 20µl, after which fluorescence was followed as a function of time. Baseline calcein release was defined as that occurring after the addition of TBS instead of the protein sample, while complete release was obtained by the addition of 10µl 10% Triton.

RESULTS

Hyper-solubilization of the lambda holin

To test the ability of GroEL to form a soluble complex with the lambda holin, S105 in 1% EBB was mixed with either GroEL, or BSA and dialyzed under conditions where detergent was quantitatively removed within 3 days. Based on results with the BR, which has 7 TMDs and is bound at 2 molecules per tetradecameric chaperonin, it was anticipated that 4 - 6 molecules of S105, with 3 TMDs (Fig. 4-1), could be solubilized by GroEL. Unexpectedly, the S protein remained quantitatively soluble in the presence of GroEL, at an 80:1 ratio of S105 molecules per GroEL, while completely
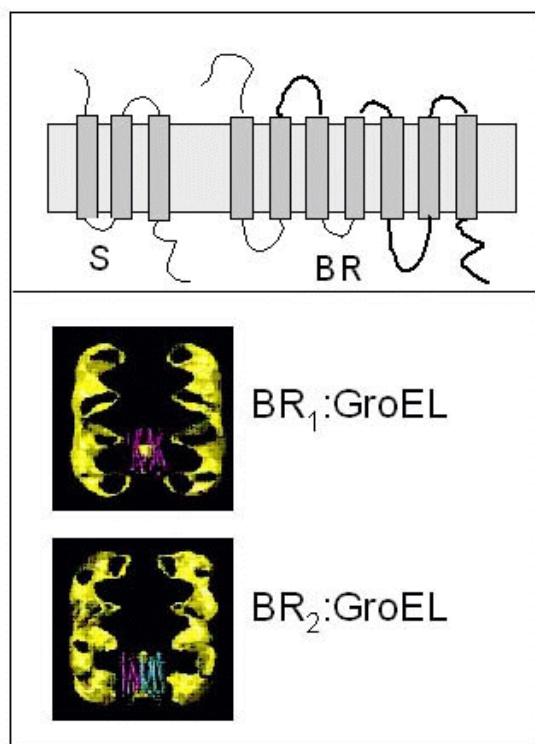
Figure 4-1. S gene products and BR: topologies and scale compared to GroEL.

Upper panel: topological model of the S proteins (S105 and S107), with three TMDs, and BR, with 7 TMDs. Lower panel: modeled images of complexes formed between one or two molecules of BR and the structure of GroEL.

precipitating in the BSA control (Fig. 4-2AB). In similar experiments performed to ascertain the limits of this GroEL-dependent solubilization of S, it was found that the S105 could be solubilized at up to 350 molecules per GroEL complex (Table 4-1; Fig. 4-3). At 11.5 kDa per S105 molecule, this level of solubilization, which we refer to as hyper-solubilization, would require complexes formed with ~ 4 MDa of holin protein per GroEL chaperonin, which has a mass of 0.8 MDa. Stored at 4degreesC, the S105 remains soluble in the presence of GroEL for at least 12 weeks at low S to GroEL ratios and 3-4 weeks at high S to GroEL ratios at 4C (not shown).

The hyper-solubilization of the functional S105 holin was also observed with the missense mutant product, S105a52v, which does not permeabilize membranes either *in vivo* or *in vitro* (Table 4-1). In contrast, the antiholin product of *S*, S107, was solubilized only at a much lower level, determined by titration experiments to be 6 molecules per GroEL tetradecamer (Fig. 4-3; Table 4-1), despite the fact that S107 differs from S105 only by the Met-Lys N-terminal dipeptide (Wang, Smith, and Young, 2000). Moreover, the presence of the antiholin prevented the hyper-solubilization of the holin (Table 4-1), mirroring the *in vivo* dominant-negative character of S107, which binds to and inhibits the lytic function of S105 (Grundling et al., 2000).
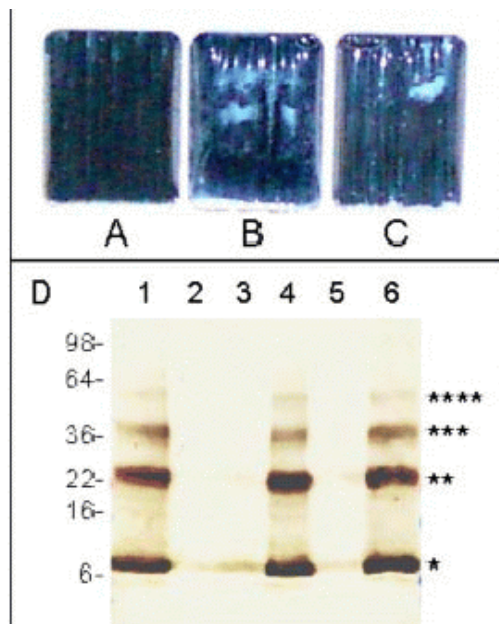
Figure 4-2. ATP-sensitive solubilization of the lambda holin.

S105 protein at 2 mg/ml in detergent was placed in dialysis chambers and dialyzed until visible precipitates formed. Panels A-C: images of chambers after dialysis. In addition to the detergent-solubilized holin, the dialysis chambers contained: A,C. GroEL (1 mg/ml); B. BSA (1 mg/ml). In panel C, the dialysis buffer contained 5 mM ATP. Panel D. S105 (100 mg/ml) dialyzed with GroEL (lanes 1,2), BSA (lanes 3,4), or GroEL + 5 mM ATP (lanes 5,6). Soluble and insoluble fractions obtained by centrifugation were analyzed by SDS-PAGE and immunoblotting with anti-S antibodies. Lanes 1, 3, 5 = supernatant (soluble fraction); 2,4,6 = pellet (insoluble fraction); Molecular masses as determined by mobility of standards are indicated. Monomer, dimer, trimer and tetramer species of the holin are indicated by asterisks.
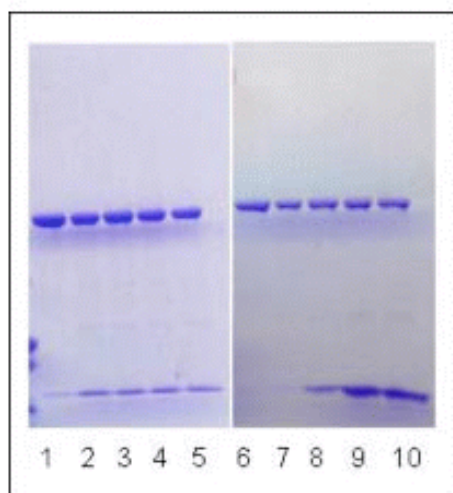
Figure 4-3. Titration of S105 and S107 into GroEL.

GroEL was mixed with was EBB-solubilized S105 or S107 at various ratios in 1 ml final volume and then subjected to dialysis to remove detergent, as described in Methods. Insoluble material was removed by centrifugation and the soluble fraction was concentrated to 0.5 ml before analysis by SDS-PAGE and immunoblotting with anti-S and anti-GroEL antibodies. The amount of solubilized S protein does not increase above 6:1 for S107 (lane 2) but increases continuously through 300:1 (lane 9) for S105. Input solute molecules per GroEL are, for S107 (lanes 1 - 5): 1, 6, 80, 160, 300. For S105, (lanes 6 - 10): 1, 6, 80, 300, 400. The position of molecular weight standards are indicated to the left.

Characterization of the GroEL-S complexes

Complexes formed at various S to GroEL ratios were analyzed by gel filtration chromatography. Using the 6:1 S105:GroEL sample, the chaperonin and the holin co-chromatographed at a position corresponding to approximately 1 MDa (Fig. 4-4A), but in the 300:1 sample, complexes containing most of the S protein eluted at a position corresponding to mass of between 2 and 6 MDa (Fig. 4-4B). Moreover, GroEL and S105 quantitatively co-precipitated with both cognate antibodies, when complexes were formed at 80:1 holin:chaperonin (Fig. 4-5). Similar quantitative co-precipitation was observed at 6:1 and 300:1 ratios of S to GroEL. Thus in both the larger and smaller types of complexes, antibodies are able to bind both solute and chaperonin. Moreover, all of the S protein in solution must be complexed with the chaperonin, and all the chaperonin is involved in complexes with S protein. However, the holin S105 and its non-lytic allelic product S105a52v can form soluble complexes with upwards of 300 holin molecules per tetradecamer, which represents more than 3 MDa of solute molecule per 0.8 MDa chaperonin, a solute mass far in excess of anything reported for GroEL. Parallel experiments with S107, which is solubilized at about 6 molecules per GroEL, showed that the only the smaller ~1Mda complexes were formed, consistent with the proposed binding capacity of one chamber of the chaperonin (Ewalt *et al.*, 1995).
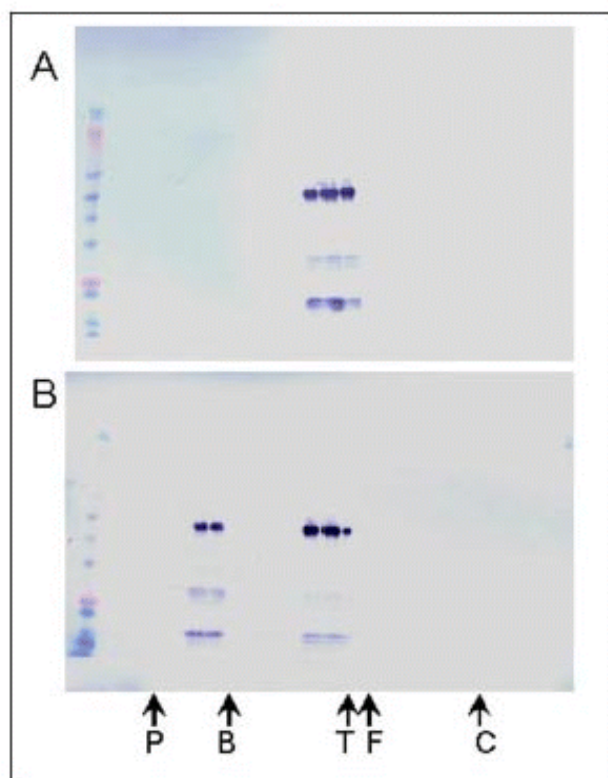
Figure 4-4. Gel filtration of S-GroEL complexes.

S105 samples dialyzed with GroEL at 6:1 (panel A) and 300:1 (panel B) were analyzed by gel filtration on a Superose 6GL column. Twenty-four 1 ml fractions were collected, concentrated and analyzed by SDS-PAGE and immunoblotting with a mixture of anti-S and anti-GroEL antibodies. The peak fractions of gel filtration standards are labeled as follows: P, Polystyrene (6 MDa); B, Blue Dextran 2000 (2 MDa); T, thyroglobulin (660 kDa); F, ferritin (440 kDa); C, chymotrypsinogen (25 kDa). SDS-PAGE standards are shown in the first lane.
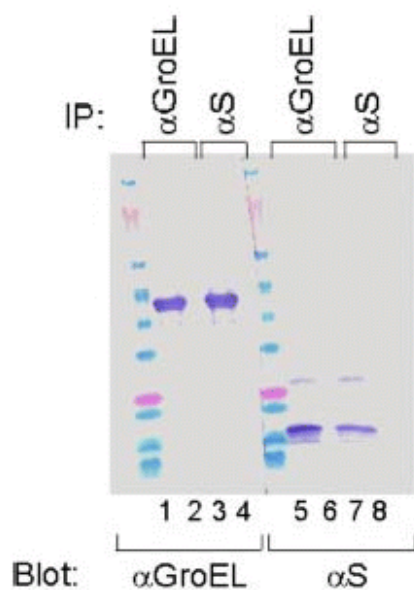
Figure 4-5. Co-immunoprecipitation of GroEL and solubilized S105

GroEL-solubilized S105 membrane protein samples were subjected to immunoprecipitation and analysis by SDS-PAGE and immunoblotting, using either anti-GroEL or anti-S antibodies as the precipitating (IP) and blotting (Blot) antibodies, as indicated. Lanes 1-4: blot with anti-GroEL; lanes 5-8: blot with anti-S. Shown are immunoprecipitates (odd numbered lanes) and supernatants (even numbered lanes) using anti-GroEL (lanes 1, 2, 5, 6) or anti-S (lanes 3, 4, 7, 8).

Molecular basis of the chaperonin-solubilization

To localize S within the S-GroEL complexes, detergent-solubilized S105 was labeled with amine-reactive Nanogold particles and subjected to dialysis with GroEL. Because of the low yield of Nanogold labeling, the loading ratio of S to GroEL was low, probably less than 1:1. Electron microscopy of GroEL loaded with S105-Nanogold conjugates (Fig. 4-6) clearly reveals the association of holin with the GroEL chaperone. Nanogold particles were consistently found within the GroEL lumen, but never around the perimeter of GroEL as deduced from face-on (Fig.4-6a-d) in conjunction with side-on projections (Fig. 4-6e and f). Face-on projections of GroEL are endowed with a 7-fold rotational symmetry, and the positions of the gold particles are close or even coincide with the center of symmetry. With the side-on projections, GroEL displays a point of 2-fold symmetry separating the two Luminal cavities; in these projections, the positions of gold particles are also indicative for Luminal binding. Nanovan staining (Yang *et al.*, 1994)aided greatly in the detection of labeled molecules. Only occasionally (Fig. 4-6f) did ammonium molybdate provide a sufficiently low contrast for nanogold to be unambiguously discriminated from protein-related densities. The background in all S105-nanogold conjugate specimens was clean. Control specimens with inactivated gold occasionally showed nanogold aggregates. However, no GroEL was labeled with gold, indicating that nanogold itself does not associate with GroEL unless conjugated to S105.

This observation is consistent with the findings of Hainfeld and Furuya (Hainfeld and Furuya, 1992), who reported a low affinity of nanogold for proteins in general. Silver-enhanced SDS PAGE revealed that only a fraction of the nanogold particles could be successfully conjugated with S105 (not shown), resulting in a low labeling efficiency. In electron microscopic analysis, less than <5% of all GroEL molecules were found to be associated with S105-nanogold. Even though the efficiency was low, all identifiable nanogold particles were found in association with GroEL molecules, which means that the labeling, albeit of low yield, is highly specific. The low yield may be due to detergent masking of the reactive lysine groups in S105. These results confirm that, at low S105:GroEL ratios, S105 is bound in the central chamber of the chaperonin (Table 4-1).

Uranyl-acetate staining of S105 solubilized at a ratio of 80:1 S105:GroEL revealed unexpected morphologies. Protein shells appear to be formed around 15 - 30% of the chaperonin complexes, suggesting a molecular basis for the hypersolubilization of the S105 holin (Fig. 4-7). Most of these shells were approximately 30 nm in diameter, or about twice the diameter of GroEL. Assuming the protein shells consist of oligomers of S105, they could contain S105 protein at about 400 molecules per tetradecamer, which would account for the overall 80:1 ratio if 20%of the GroEL molecules are involved and the rest carry S105 at about 6 molecules per tetradecamer, as in the case of S107.

Table 4-1. Solubilization of the lambda holin by GroEL

Protein[1]	Input ratio[2]	% Soluble[3]	# experiments[4]
S105	1:10	100	5
	1:1	100	50
	10:1	100	10
	50:1	100	5
	80:1	100	>100
	100:1	100	5
	150:1	100	5
	200:1	100	5
	250:1	100	5
	300:1	100	10
	350:1	100	10
	400:1	75-80	2
+ATP (5mM)	80:1	0	5
+ADP (5mM)	80:1	slow precipitation	2
+AMP (5mM)	80:1	100	2
+AMPPNP (5mM)	80:1	0	1

Table 4-1. (continued)

Protein[1]	Input ratio[2]	% Soluble[3]	# experiments[4]
S105a52v	1:10	100	5
	1:1	100	50
	10:1	100	5
	50:1	100	5
	80:1	100	>100
	100:1	100	5
	150:1	100	5
	200:1	100	5
	250:1	100	10
	300:1	95	10
	350:1	80	5
	400:1	50	2
+ATP (5mM)	80:1	0	2
+ADP (5mM)	80:1	slow precipitation	2
+AMP (5mM)	80:1	100	2
+AMPPNP (5mM)	80:1	0	1

Table 4-1. (continued)

Protein[1]	Input ratio[2]	% Soluble[3]	# experiments[4]
S107	1:10	100	2
	1:1	100	10
	3:1	100	10
	5:1	100	10
	6:1	100	10
	20:1	<40	3
	50:1	<15	3
	100:1	<10	2
S105 & S107 (2:1)	1:10	100	2
	1:1	100	2
	3:1	100	2
	10:1	<75	2
	100:1	<15	2

[1]Input protein samples in the detergent EBB, as specified in Experimental Procedures. GroEL present at 100 mg/ml.
[2]Ratio of solute protein molecules to GroEL.
[3]Percent soluble protein after dialysis, estimated from intensity of immunoblot bands, standardized by purified protein.
[4]Number of trials conducted

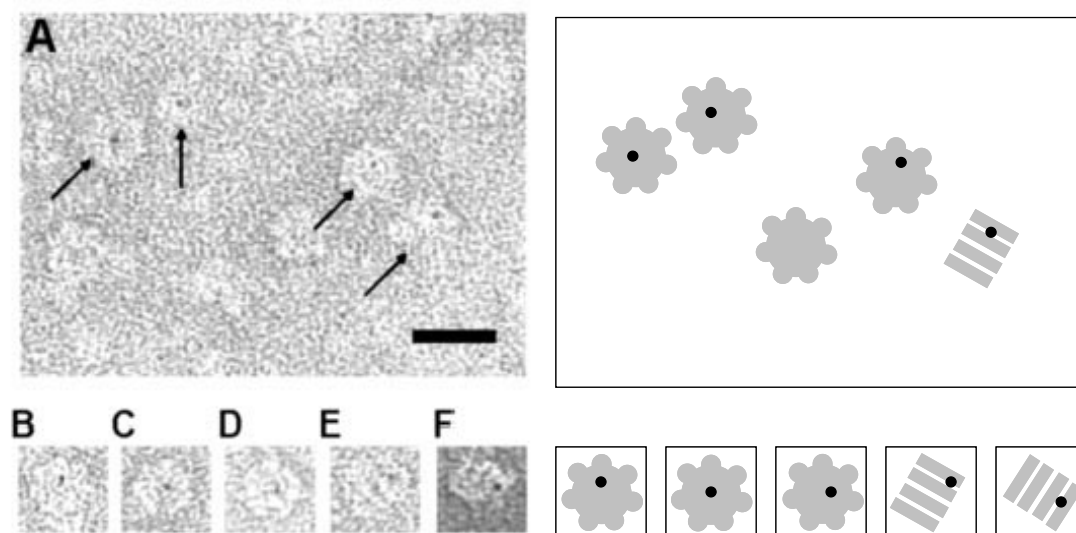**Figure 4-6. Electron micrographs of negatively stained S105-nanogold conjugates in the presence of GroEL.**

GroEL molecules labeled with S105-Nanogold can be clearly discerned and are highlighted by arrows in the overview (A) and individually boxed molecules (B-F). Note that (A-E) were stained with methylamine vanadate (Nanovan), (F) with ammonium molybdate. The scale bar corresponds to 20 nm.

When S105 was loaded into GroEL at 1:1 or 2:1,shells were not observed (not shown). Also, chains of GroEL molecules were occasionally observed at the higher input ratios of S105 to GroEL, but not at low input ratios (not shown), suggesting that S105 molecules could form mixed oligomers with the tetradecamer.

S105 delivered from GroEL complexes is functional irrespective of the loading ratio.

BR solubilized by forming complexes with GroEL was shown to retain its normal conformation, as judged by its spectroscopic characteristics and could be delivered efficiently and in functional form to artificial membranes, as judged by the ability to energize the membranes upon exposure to light (Deaton, *et al.* 2004b). Although there is no independent measure of the conformational state of the S protein in complexes with GroEL, it is possible to assess its function by using a membrane permeabilization assay. GroEL-S complexes were prepared at both high and low ratios of S to GroEL, and the large ~4MDa complexes were purified by gel filtration, as shown in (Fig. 4-4). Both types of S-GroEL complexes were shown to effect dye release from liposomes, whereas in controls done with GroEL alone or GroEL loaded with the S105a52v non-lytic allelic product there was no significant dye release above background (Fig. 4-8). Moreover, the rate of permeabilization of liposomes is clearly much higher for the large complexes than for the smaller complexes, with comparable levels of S105. Thus the large complexes, although clearly carrying S protein far in
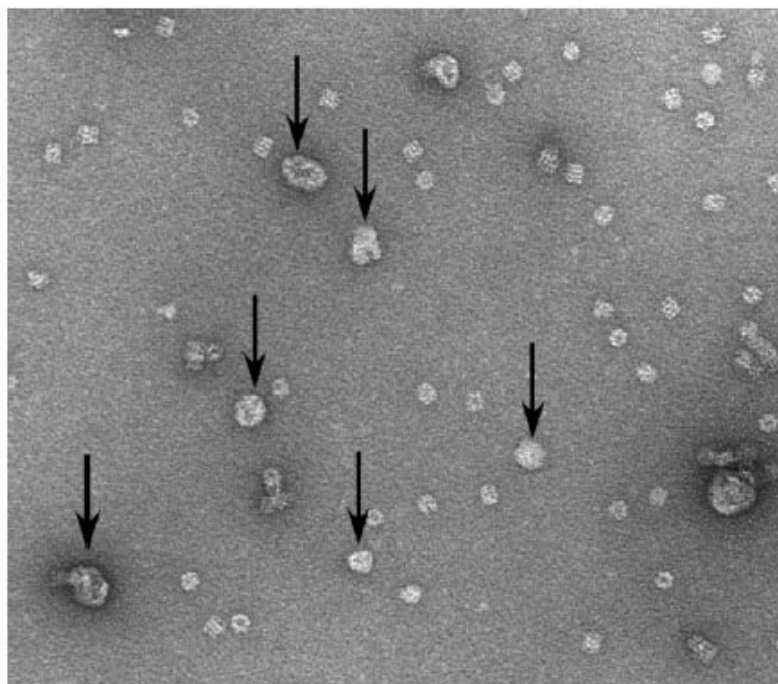
Figure 4-7. S105 forms shells around GroEL molecules.

Arrows indicate structures found in S105 and GroEL mixtures, dialyzed from detergent at an S105:GroEL ratio of 80:1. Free GroEL molecules are readily discerned. The structures were 20-30 angstroms on average.

excess of the binding capacity of the chaperonin chambers, consist of S protein capable of inserting efficiently into artificial membranes and then proceeding to form holin lesions.

DISCUSSION

Hyper-solubilization of the holin S105 and its application to the study of holin function.

In a previous report, we demonstrated that BR could be complexed with GroEL and then transferred efficiently to pre-formed membranes (Deaton *et al.*, 2004a) providing a new approach for the study of integral membrane proteins. This method is particularly attractive for the study of bacteriophage holins, which accumulate harmlessly in the host membrane until suddenly triggering and disrupting the membrane, an event that terminates the viral life cycle. Clearly this "hole formation" event cannot be studied if proteoliposomes containing holins are formed by traditional methods involving removal of detergent from mixtures of solubilized lipid and protein. Thus it was gratifying that the protein products of the lambda holin gene *S* were found to retain solubility after removal of detergent in the presence of GroEL. Surprisingly, however, the amount of the lethal holin S105 which could be solubilized was in excess of 300 molecules of S105 per GroEL. In contrast, titration experiments showed that the antiholin S107, differing from S105 only by the N-terminal two residues Met-Lys, was
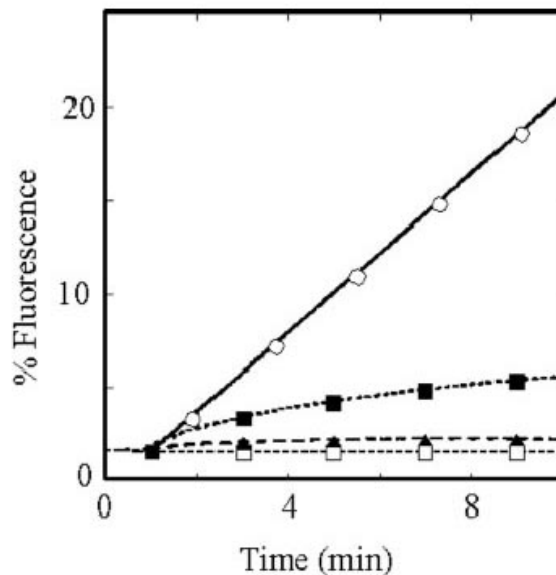
Figure 4-8. S105 delivered from GroEL complexes is functional for membrane permeabilization.

Lambda holin protein solubilized at low (6:1) or high (300:1) ratios of *S* gene product to GroEL was added to pre-formed liposomes loaded with calcein, and the release of dye monitored by fluorescence. The large complexes were purified by size exclusion chromatography. Protein added at t = 1 min. 100% fluorescence corresponds to the value after addition of detergent. Open circles: S105, 300:1 (7 mg/ml S105, 1.7 mg/ml GroEL). Solid squares: S105, 6:1 (5mg/ml S105, 50 mg/ml GroEL). Solid triangles: S105a52v, 6:1 (10 mg/ml S105a52v, 100 mg/ml GroEL). Open squares: GroEL only (200mg/ml).

solubilized up to a limit of 6 molecules per GroEL, approximately what would be expected from the estimated capacity of the chaperonin chamber (Ewalt *et al.*, 1997; Song *et al.*, 2003). S protein solubilized by GroEL in this way is stable for weeks at 4C, irrespective of the loading ratio. Moreover, the allelic state of the S105 protein was irrelevant, since the S105a52v product, which is non-lytic *in vivo*, was also hyper-solubilized by GroEL. Finally, the hyper-solubilized material is at least as effective, on a per S molecule basis, as the complexes formed at low ratios, in terms of delivery to and permeabilization of liposomes.

Two types of ATP-sensitive complexes formed between S protein and GroEL

Gel filtration analysis of the S105-GroEL solutions formed at high S105 to GroEL ratios revealed that most of the holin was in complexes of ~4 MDa, the largest GroEL-protein complexes yet reported. In contrast, complexes formed at low ratios of S105, or between S107 and GroEL, eluted at the same position as GroEL alone, as expected since the molecular weight of S105 or S107 is only about 11.5K, compared to the 840K of the chaperonin. Visualization of the S105-GroEL complexes by electron microscopy revealed that, in the samples formed at high ratio, the chaperonin appeared to be completely covered with protein, presumably a shell of S105 molecules. In contrast, complexes formed at low levels of S protein per chaperonin contain S only in the chamber of the tetradecamer, as judged by nanogold staining. Both types of complexes are sensitive to ATP. A reasonable model, then, for these complexes is that

the hydrophobic surface of the apical domain of the GroEL subunits is required for the binding and solubilization of the S protein solute. In the current model for the GroEL-GroES cycle for refolding of denatured proteins, this surface is exposed alternately on each heptameric sub-complex of the tetradecamer (Roseman *et al.*, 2001). The fact that ATP binding instantly releases the bound membrane protein solute is strong evidence that the restriction to single cavity loading reflects the conformational dynamics of GroEL. ATP binding is known to cause a conformational change which is thought to cause internal rotation of the apical domain for all of the subunits of one heptamer. The fact that the shell complexes are also sensitive to ATP suggests that the foundation of the shell is a complex with S protein bound in the chamber of GroEL, and that there are interactions between the chamber-bound and external S proteins which are necessary for the stability of the complex.

In both complexes, GroEL and S are accessible to their respective antibodies. This suggests that the S105 shells are not rigid assemblies which would block access of antibodies to GroEL. A substantial number of GroEL molecules lacking shells are present even at the maximum S105 to GroEL loading, as judged by gel filtration analysis and electron microscopy. The shells may be in dynamic equilibrium, with complexes exchanging S105 protein with one another, and occasionally segregating chaperonins lacking the protein shell. Nevertheless, the immunoprecipitation experiments show that all of the GroEL molecules contain S105 under these conditions, even if not all of them

carry S105 shells.

The *S* gene is expressed at relatively low levels *in vivo*, such that on the order of 10^3 molecules of total *S* gene product are present per cell at the time of lysis (Chang, Nam, and complexes formed at high input ratio of S105 to the chaperonin are not indicative of complexes that might form *in vivo*. Nevertheless, in view of the fact that the hyper-solubilization effect Young, 1995). Considering the much larger numbers of GroEL molecules in the cytosol, the large occurs with S105 but not with S107, clues to the physical differences between the holin and antiholin might be deduced from consideration of models for the structure of the large complexes. The gross dimensions and apparent molecular mass suggests that upwards of 300 S105 molecules polymerize in shells around S105-saturated GroEL tetradecamers. Since the large complexes can be destroyed by the addition of ATP, it is likely that these shells are connected to the chamber-bound S105 molecules. Models of the growth of the shell from complexes with S proteins bound in the chamber are shown in (Fig. 4-9).

Whether there is S105 in both chambers is unknown, although preliminary results from negative-stain single particle analysis suggests that extra density is onlyfound in one chamber, not only for S105 but also for other integral membrane proteins like BR and LacY (J. Sun, C. G. Savva, J. F. Deaton, H.R. Kaback, M. Svrakic, R Young, and A. Holzenburg, submitted). Irrespective of whether both chambers are
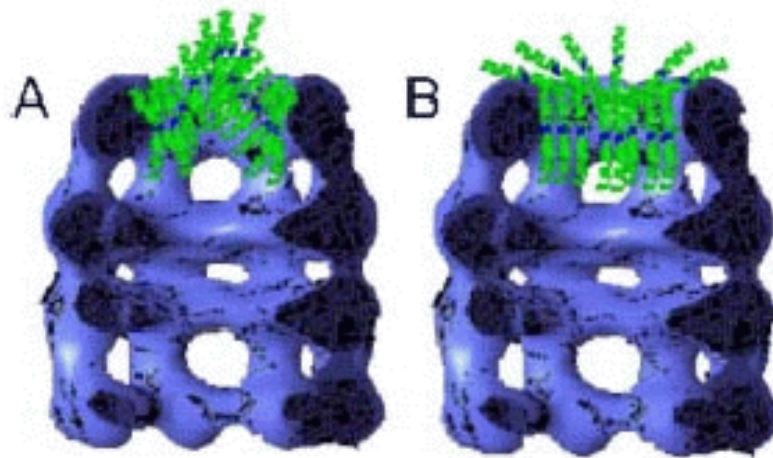
Figure 4-9. Models for formation of S-GroEL complexes.

A. The TMDs of the S105 protein associate with each other within the GroEL chamber.
The oligomers that form are oriented so that continued polymerization beyond the
GroEL chamber is possible via TMD-TMD interaction. B. The hydrophobic faces of
one or more of the S105 TMDs bind to the hydrophobic surface of the apical domain of
GroEL. The hydrophilic surface of TMDs 1 and/or 3 are exposed to the opening of the
GroEL chamber and serve as nucleation sites for the polymerization of S105 around the
outside of the GroEL molecule.

loaded, more than 300 S molecules must be present in the external shell. Assuming 300 S105 molecules are aligned by TMD-TMD interactions to make up the surface of a 30 nm large complex, the density of S105 would be ~ 0.4 molecules/nm^2, approximately what would be expected from tight helical packing of the S105 molecules, each with 3 TMDs. S105 oligomerizes to a high degree as part of its function in the timing of host lysis (Grundling, Blasi, and Young, 2000c), so this extraordinary mostly two-dimensional polymer might reflect the ability of GroEL to maintain S105 in a polymerization-proficient conformation similar to that it assumes in the bilayer. Indeed, the S105 protein in these complexes is even more efficient at permeabilization of liposomes than S105 bound at low ratios to GroEL (Fig. 4-8). The fact that the S105a52v protein also forms the large complexes might be taken as evidence against this model. However, the two-dimensional concentration of S protein *in vivo* is never more than 10^{-4} molecules/nm^2 of membrane, more than 10^3-fold less than in these putative shells. Thus, with the A52V missense change, a TMD-TMD interaction defect which would block polymerization *in vivo* by changing affinity by up to two orders of magnitude would not be relevant at the high loading ratios *in vitro*. In contrast, the limit of 6 molecules of the antiholin S107 per chaperonin approximates the putative binding capacity of the GroEL cavity. Significantly, mixtures of S107 and S105 also form only the small complexes, indicating that binding is restricted to the cavity. This dominant-negative effect parallels the lysis-inhibiting function of the S107 and suggests that the antiholin may function by blocking high-level polymerization *in vivo*. GroEL as a vector

 for delivery of integral proteins to bilayers.

With the results presented here and previously (Deaton *et al.*, 2004a), GroEL has been shown to have the ability to deliver BR and both the holin and antiholin products of the lambda S gene to liposomes rapidly, efficiently, and in functional form. These findings support previous suggestions that GroEL may have a role in the post-translational localization of integral membrane proteins *in vivo*. In any case, the important practical implications for the study of membrane proteins in their native environment is, alone, sufficient justification for further study of this phenomenon. In particular, the fact that neither ATP nor the co-chaperonin GroES is required for release of the cargo into the bilayer of the liposome is intriguing. The C-terminal region of each GroEL subunit has affinity for membranes *in vitro* (Torok *et al.*, 1997). The binding of GroEL loaded with a membrane protein to the surface of the bilayer may be associated with conformational changes analogous to those observed during the GroES-ATP binding cycle for GroEL loaded with denatured soluble proteins; the liposome system described here should be suitable for investigating this possibility. In any case, the ability to deliver both BR, with its light-dependent proton-pumping ability, and *S* gene products to artificial membranes efficiently, it is now feasible to study the function of holins in the membrane environment and, importantly, the dependency of hole-formation on the energy state of the membrane. Attempts at reconstitution of the entire holin-mediated lysis triggering event, including the function of the antiholin, are now underway.

CHAPTER V

CONCLUSIONS

IN VITRO CHARACTERIZATION OF THE LAMBDA HOLIN

Purification and physical characterization of the lambda holin

Typically, the first step in the purification of an integral membrane protein is its removal from the membrane by detergent extraction. The choice of detergent is largely empirical and is influenced by both the anticipated purification scheme and the types of experiments in which the purified protein will be employed. The ideal detergent for the purification of the lambda holin would (1) allow its quantitative extraction from the inner membrane of *E.coli*, (2) maintain the holin in solution during extended periods of storage, (3) not interfere with purification of hexahistidine-tagged forms of the holin by immobilized metal affinity chromatography (IMAC), and (4) be easily removed by dialysis. Of the numerous nonionic, ionic, and zwitterionic screened, only the nonionic detergent Empigen BB met these four criteria. Empigen BB was clearly superior to octyl-glucoside which had been previously used for holin purification in that the Empigen BB-solubilized holin protein remained soluble for months while the same protein solubilized in octyl-glucoside precipitated within 24 hours. Moreover, the lambda holin in Empigen BB was found to have a CD spectrum that was consistent with presence of three transmembrane segments. This is in stark contrast to the octyl-glucoside solubilized protein which had previously been shown to have only two

transmembrane segments using the same methodology. Finally, CD analysis of the lambda antiholin protein and various nonfunctional forms of the lambda holin, all in Empigen BB, revealed that they all had secondary structures that were indistinguishable from that of the functional holin. Thus, neither the two residue amino-terminal extension that converts the holin into an antiholin nor the many missense mutations which inactivate the holin cause gross changes in the secondary structure of the protein. This is not surprising since the amino-terminal extension which converts the holin into an antiholin is thought to affect, primarily, the topology of the protein (see below) while the inactivating, missense changes probably act by affecting the packing of the transmembrane segments in the membrane. In neither case would secondary structure be necessarily affected.

The holin and antiholin of lambda have different topologies

The only difference between the two proteins produced from the S gene was the two additional amino acids on the n-terminus of the S107 protein. The two additional amino acids though greatly alter the phenotype of the protein and has been shown here the topology of the protein in the inner membrane. The difference in sequencing S107 versus S105 by edman degradation suggest that S105 quickly inserts into the inner membrane after translation. S107 inserts transmembrane domains two and three (probably as a helical hairpin), while requiring an additional component/event to translocate the n-terminal domain across the membrane. This event is probably tied to

the energy state of the inner-membrane, due to the fact the holin and antiholin can be triggered by energy poisons (Smith, Struck, et al. 1998). The two additional positive charges of S107 could hinder translocation because of both the hydrophobic nature of the membrane and the negative charge of the inner leaflet of the inner membrane due to the proton motive force and the negatively charged lipid head groups. The initial triggering process (which could occur from enough S105 homo-oligomerizing) that the membrane potential collapses, thus releasing S107 n-terminal to translocate across the membrane. This process would free up not only S105 but also S107 which would now adopt the same topology of S105, more than doubling the S protein for lesion formation.

Improvements in the *in vitro* assay for the lambda holin

Previously, a dye release assay was designed to look at the activity of the lambda holin *in vitro*. Several steps were taken to improve the experiment. The first modification was to mirror as closely as possible the total lipid head group charge of the inner membrane of *E.coli*. The exchange of total non-ionic lipids to a mixture of 70% non-ionic and 30% anionic lipids increased the amount of dye that was released from the liposomes by S. Since *E.coli* is a major host for lambda and S has a highly positive charged C-terminus, it was not surprising that S was more efficient with the addition of negatively charged lipids. The negative head groups could help anchor the C-terminus of S to the membrane and perhaps enhance insertion of S into the liposomes. The experiments still required S to be delivered from diluting the protein out of a chaotrope

(due to solubilization of the liposomes by detergents). This method required S to go from an unfolded state into an aqueous buffer where S could either insert into liposomes or precipitate out of solution. Early experiments showed that most of S precipitates, requiring a more efficient method of delivery of S to the liposomes. The optimization of deliver of S to liposomes lead to the following discoveries.

A new method to solubilize membrane proteins

The efficient, non-stoichiometric solubilization of membrane proteins by GroEL is unexpected, remarkable, and potentially very useful in many applications that currently require detergents or chaotropes. For these reasons, this phenomenon deserved thorough characterization. This work was a quantum leap in studying membrane proteins, which required a hydrophobic environment to stay soluble. This work provided a new method to deliver functional membrane proteins to lipid bilayers that was required to fully study and understand the structural and functional characteristics of membrane proteins. The past methods of reconstituting membrane proteins were difficult and could not be accomplished for many proteins such as the lambda holin. The method of reconstituting membrane proteins in lipid bilayers by solubilizing each in detergent, mixing them and removing the detergent, created non-uniform liposomes or failure to form proteoliposomes at all. In cases of membrane proteins that disrupt lipid bilayers, the membrane protein destroys the proteoliposomes as soon as they are made. Dilution of the detergent until it no longer disrupted lipid bilayers is also a common method to

reconstitute membrane proteins in lipid bilayers. The main problem with this technique is the dilution is far below the critical micelle concentration (CMC) of a detergent, which usually causes the membrane protein to precipitate. Finally the membrane protein can be reconstituted into a chaotrope then added to preformed liposomes. The problems with this technique was that the proteins are unfolded and must then be diluted into an aqueous buffer, bind to the liposomes and finally insert/fold in the liposomes. This process was not very efficient, resulting in the majority of the membrane protein forming an insoluble aggregate. The ability of GroEL to form soluble complexes with membrane proteins allows them to be delivered to preformed lipid bilayers which can be used to study the structure or function of the protein. The assays include liposomes, inverted membrane vesicles (INV) and planar lipid bilayers. All of these assays are common methods to study membrane proteins, yet haven't been used for many membrane proteins due to the difficult conditions mentioned above.   Furthermore, since the structure of GroEL is known, if GroEL soluble membrane protein complexes could crystallize, the membrane proteins structure could be solved using particle displacement. Another technique that could be useful for crystallography is based off the fact that the GroEL soluble membrane protein complexes are ATP sensitive, resulting in a precipitation of the membrane protein in the presence of saturating ATP concentrations. The addition of saturating ATP to GroEL membrane protein complexes under crystal screens, could result in an ordered precipitation of the membrane protein, which would

produce crystals. This technique for crystals could lead to many more membrane protein structures determined by x-ray crystallography. In conclusion, the ability of membrane protein to form soluble complexes with GroEL, allows the use of numerous assays that were unavailable for membrane proteins previously.

A new role for GroEL in *E.coli*?

This work could lead to a better understanding of the role of GroEL *in vivo*, especially in the context of membrane proteins. GroEL is known to help fold soluble proteins, although in most cases this activity requires the co-chaperonin GroES and ATP (Ellis, 2003). Moreover, the GroEL tetradecameric complex has been shown to interact with artificial membranes by virtue of a small, hydrophobic domain at the C-terminus of the monomer (Torok *et al.*, 1997). However, little has been done with GroEL interactions with membrane proteins, *in vivo* or *in vitro*. Interestingly, in the description of the original groEL altered-function mutants in 1973 (Georgopoulos and Eisen, 1974), it was shown that phage lambda infections exhibited delayed lysis phenotypes. In light of our more recent work demonstrating that the S protein is the sole determinant of the timing of lysis, this old and overlooked result suggests that S may interact with GroEL *in vivo*. Overexpression of GroEL during the lytic cycle of several different holins, lead to a change in lysis timing, which is entirely determined by the holin. Excess GroEL showed a delay in lysis for lambda S and S21 holin mediated lysis, perhaps indicating that excessive GroEL is competing out holin that is inserting into the membrane , thus

delaying lysis. Whereas with the P2 holin, excess GroEL increases the lysis timing by more than forty minutes, indicating perhaps translocating or insertion of the holin is more efficient at higher levels of GroEL. Moreover, this could indicate that GroEL has a general function, independent of GroES, for helping integral membrane proteins insert into the membrane. Given that GroEL is conserved in all cells and in organelles with independent protein-synthesizing machinery, the implications of this new function may be profound.

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