ABSTRACT

Title of Thesis: SITE-DIRECTED MUTAGENESIS OF GROEL: DEVELOPING A SYSTEM FOR MONITORING ALLOSTERIC MOVEMENTS BY FLUORESCENCE RESONANCE ENERGY TRANSFER

Yu Yang, Master of Science, 2006

Thesis directed by: Professor George H. Lorimer
Department of Chemistry and Biochemistry

The *Escherichia coli* chaperonin protein GroEL can assist protein folding to its native state through the consumption of ATP. Accompanying this process, GroEL undergoes structural change, resulting in an expansion of the central cavity. Monitoring apical domain movement by fluorescence resonance energy transfer (FRET) between two mobile apical fluorophores, can provide information about the GroEL allosteric transitions. To reach this goal, the three native cysteine residues on each subunit of wild type GroEL were removed and a new cysteine site in the apical domain was introduced by site-directed mutagenesis. Fluorescent probes were attached to the cysteine residues, allowing us to perform FRET experiments. The observed change of FRET efficiency (E) reported the GroEL structural changes.

SITE-DIRECTED MUTAGENESIS OF GROEL: DEVELOPING A SYSTEM FOR MONITORING ALLOSTERIC MOVEMENTS BY FLUORESCENCE RESONANCE ENERGY TRANSFER

By

Yu Yang

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2006

Advisory Committee:

Dr. George H. Lorimer, Chair
Dr. Dorothy Beckett
Dr. Douglas Julin

ACKNOWLEDGMENTS

The following thesis was completed through the insights and direction of several people.

Firstly, deep thanks to my mentor, Dr. George Lorimer, whose encouragement, scientific attitude, patience and financial support guided me during my graduate study, the research and writing of the thesis. His kindness made my experience in this lab a happy one. I am personally very fortunate to have him as my graduate supervisor.

Secondly, I thank my committee members, Dr. Beckett and Dr. Julin, who taught me the required courses that prepared me to commence this thesis.

Special thanks to Sarah C. Wehri. For me, she is a classmate, friend, colleague and teacher. For many fluorescent experiments, she guided me, closely looked at the results and gave me lots of useful suggestions. She also served as the first editor for this thesis with critical thoughts. Without her, it would not have been possible to complete this thesis. Her help is highly appreciated.

In addition, I thank Dr. Asha Acharya, who used her scientific experience to solve my problems in the lab. She also was the reader for this document to check English grammar and style. She's a friendly colleague. Thanks for her help and valuable hints.

I would like to thank Patrick Kates, who taught me the necessary techniques in this lab in the beginning. I also thank Patrick Schmidlein, Seth Yandrofski and Zhechun Zhang for their friendship.

Finally I would like to thank my family for their support. My parents, Yunmei Yang and Dengfeng Li, were always giving me encouragement and strength. My husband Dongyu Guo supported me deeply. His patience, love and encouragement helped me to

complete this project. Thanks to my daughter Jie Guo for her deep understanding and love and my son Ryan Yang Guo's cooperation. I am truly thankful to have them in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α-LA	α-lactalbumin (bovine)
E	fluorescence resonance energy transfer efficiency
FPLC	fast protein liquid chromatography
FRET	fluorescence resonance energy transfer
GroEL(wt)	wild type GroEL containing three cysteines at 138, 458 and 519
GroEL(cf)	cysteine free GroEL
GroEL(sr)	single ring GroEL containing R452E, E461A, S463A and V464A at the equatorial plate
Rubisco	ribulose bisphosphate carboxylase
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SP	substrate protein

Chapter 1

Introduction

1.1 Why does protein folding require GroEL

Functional proteins have their characteristic unique three-dimensional conformations or structures (1). The protein's linear sequence of amino acids contains all the structural information required for it to fold to its biologically active state (32). The accurate transfer of information from DNA to protein depends on the cell's ability to perform a complex process at high speed with no mistakes. For some proteins, correct spontaneous folding does not occur *in vivo*, so the process requires error corrections. Cells have developed some proteins to assist other proteins to fold properly (32). These assisting proteins are called chaperonins.

The chaperonins are a subgroup of molecular chaperones, the best studied of which in mechanism and structure is *Escherichia coli* GroEL (hsp60) (49). It is thought that GroEL binds nonnative polypeptide substrate via hydrophobic interactions. In the presence of co-chaperonin GroES and ATP, the polypeptide substrate is enclosed in a microenvironment that is thermodynamically favorable for correct protein folding. Upon hydrolyzing ATP, the properly folded polypeptide is released from the chaperone (58). Most SPs require multiple rounds of binding and release.

1.2 Basic structure of GroEL

GroEL is a homo-oligomer with 547 amino acids (22). It contains 2 rings stacked back to back (39). Each ring is composed of seven identical 57-kDa subunits (47) (Figure 1-1).

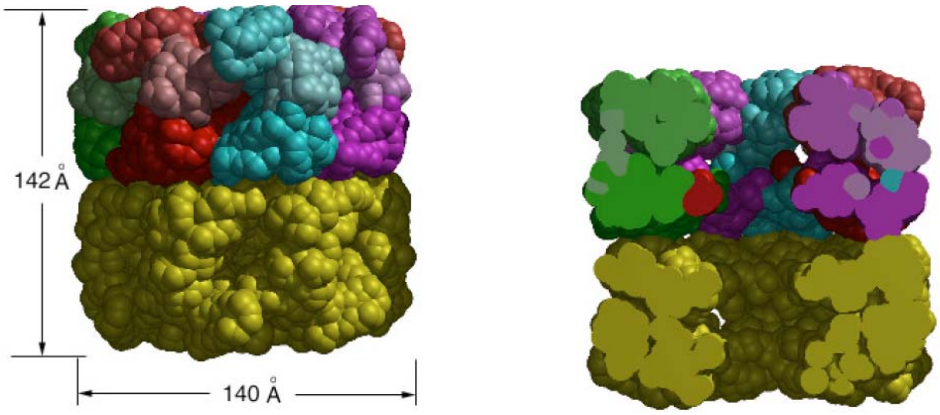


Figure 1-1. Overall architecture and dimensions of GroEL. van der Waals space-filling models (6Å spheres around C) of GroEL. Left is outside view, showing outer dimension; Right shows the inside of the assembly and was generated by slicing off the front half with a vertical plane that contains the cylindrical axis. Various colors are used to distinguish the subunits of GroEL in the upper ring. The domains are indicated by shading: equatorial, dark hue; apical, medium hue; intermediate, light hue. The lower GroEL ring is uniformly yellow. (Figure from Sigler, Xu et al. 1998).

Each GroEL monomer contains three distinctive domains (56):

1). The equatorial domain provides residues for the inter- and intra-ring interactions of the protein complex and contains the ATPase site on the inner sides of the GroE cylinder.

2). The apical domain is less well organized and more locally flexible than the equatorial domain. It contains the SP and GroES binding sites.

3). The intermediate domain connects the equatorial domain and the apical domain (20,47).

The crystal structure of GroEL has been determined to 2.8 Å (4). GroEL is a hollow, thick-walled cylinder 135Å in diameter and with height of 145Å, containing a central cavity. The electron microscopy indicated that the GroEL oligomeric complex is

composed of two seven-subunit rings, arranged with nearly exact sevenfold rotational symmetry (47).

1.3 Basic structure of GroES

GroES is composed of 7 identical 10-kDa subunits (Figure 1-2).

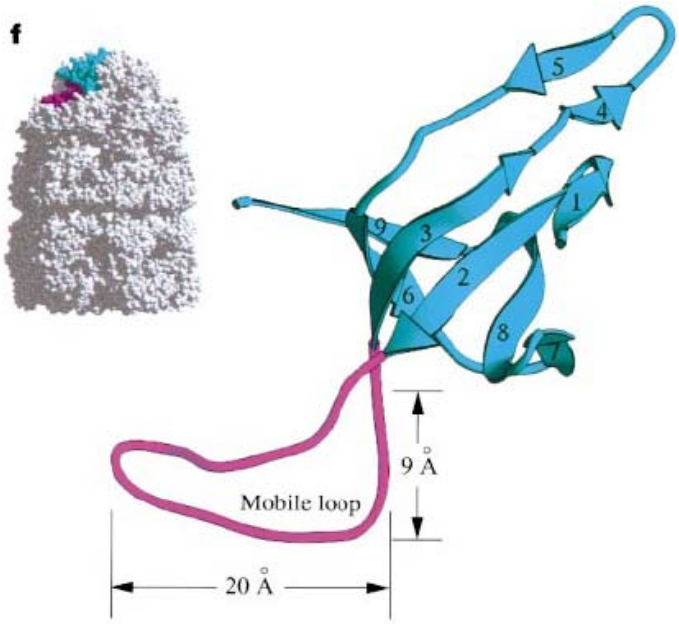


Figure 1-2. Ribbon drawing of one subunit in the GroES ring. (Figure from Xu 1997).

The crystal structure of GroES displays a dome-shaped architecture with outside dimensions of 70-80 Å in diameter, a height of 30 Å. The inside dimensions measure 30 Å in diameter and 20 Å in height (20). A core β-barrel structure with two β-hairpin loops is found in each of the seven subunits. One of the two β-hairpin loops stands upward and inward at the top of the dome, enclosing the structure. The other is a disordered and unstructured mobile loop (Glu 16 to Ala 32) at the bottom of the GroES heptamer. The second loop is implicated in GroEL-GroES binding.

1.4 GroEL/ES complex structure

In general, GroES binds to GroEL with 1:1 ratio (1 $GroES_7$ per 1 $GroEL_{14}$). The asymmetric $GroES_7$-$GroEL_{14}$ complex (Figure 1-3) is referred to as "bullet" (46).

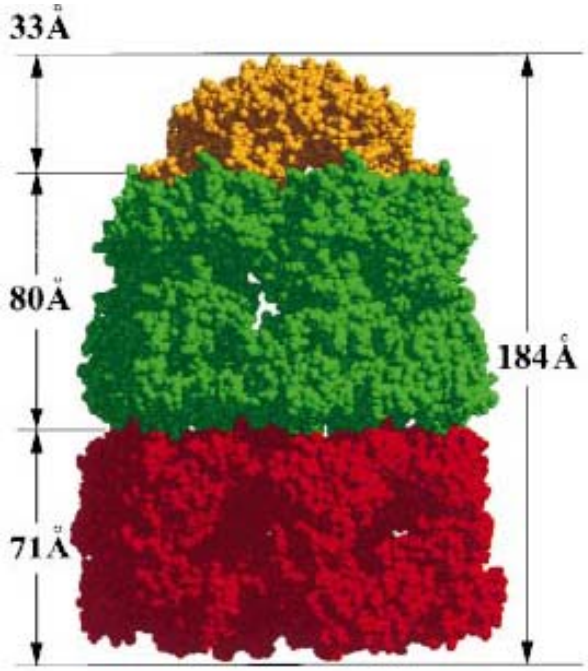


Figure 1-3. Overall architecture and dimension of the GroEL–GroES "Bullet" complex. van der Waals space-filling model of the entire complex in a side view . The complex is colour coded as follows: trans GroEL ring, red; cis GroEL ring, green; GroES, gold. (Figure from Xu & Horwich et al. 1997).

The symmetric complex $GroES_7$-$GroEL_{14}$- $GroES_7$ is referred to as a "football" (46). Even though the role of the "football" complex in the GroE reaction cycle is disputed, several hypotheses have provided an explanation for its involvement in SP folding (2,12).

1.5 The GroE reaction cycle

It was found that ATP, Mg and K^+ are very important and necessary effectors in the GroE cycling mechanism (18,52). The GroEL reaction cycle (Figure 1-4) is described as following:

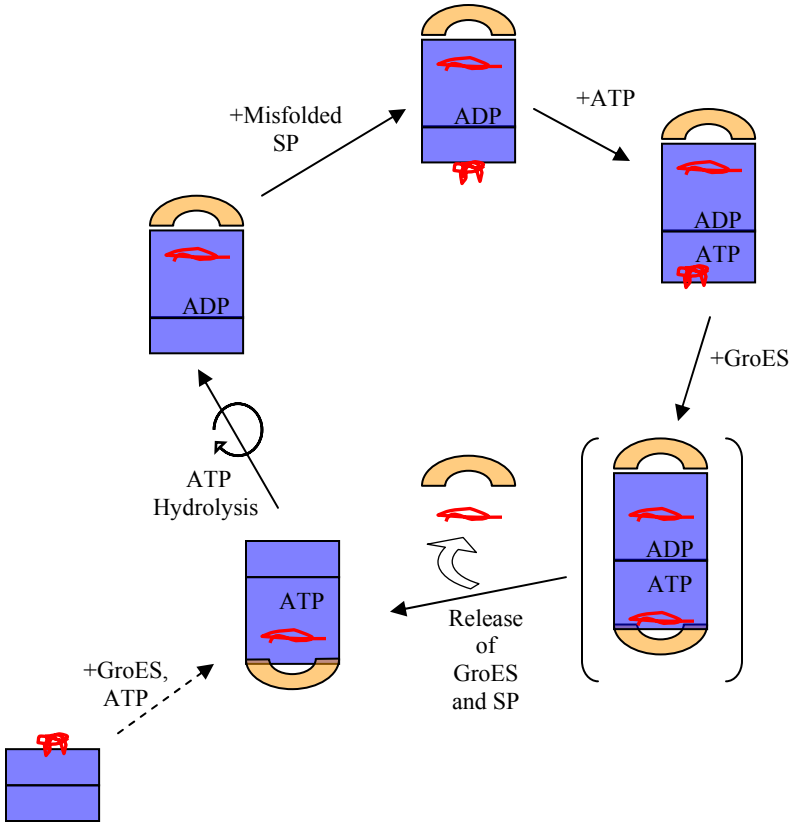


Figure 1-4. The GroE Reaction Cycle. Blue is GroEL, orange GroES, red SP (SP). The events depicted are described in the text. Bracket means this "football" which is unclear for its role in the cycle. (Figure from Grason 2003).

1.5.1. SP binding

GroEL facilitates a wide variety of unfolded or partially unfolded proteins to fold correctly. Binding of non-native protein to GroEL also prevents aggregation. GroEL has no affinity for native SPs. Without chaperonin non-native subunits of Rubisco aggregate,

whereas in the presence of GroEL a stoichiometric complex was formed that facilitated the production of native Rubisco upon addition of ATP/GroES (49).

Substrates cannot be exchanged across the equatorial plane between the two cavities. The polypeptide binding sites lie at the inner top rim of the apical domains. The sites necessary for polypeptide binding have been identified by mutational analyses (15). Nine residues in helices H and I and a loop between strand 6 and 7 are indispensable. Nonpolar side chains from eight of the nine residues face the central cavity in the unliganded GroEL structure (15). Therefore, along the inner edge of its apical cavities GroEL exhibits a ring of hydrophobic binding surface. The role of hydrophobicity in polypeptide binding has been examined and confirmed not only from the standpoint of GroEL but with a number of SPs (31). A negative heat capacity change was detected by isothermal titration calorimetry when a stably unfolded version of substilisin bound to GroEL, indicating occurrence of hydrophobic interactions. Other experiments also conclude that a maximum exposure of hydrophobic surface in nonnative peptide favors its binding to GroEL (53). Besides the hydrophobic interactions, to some extent electrostatic interactions also play role in substrate binding (37).

The volume of the GroEL cavity is limited to SPs of less than 70 kDa. The access size for nonnative polypeptides would be slightly smaller. However, proteins larger than 100 kDa may form stable binary complexes with GroEL because at this stage of binding a substantial part of bound peptide protrudes outside the cavity (49). A pair of parallel α-helices of the apical domain form a flexible groove in which polypeptides bind. This groove is different in the structure of unliganded GroEL, GroEL/peptide complexes, or GroEL in complex with GroES and ADP (57).

1.5.2. Nucleotide and GroES binding

The binding of MgATP to the active site in the equatorial domain of GroEL triggers a series of concerted, rigid-body, domain movements that are amplified in the presence of GroES (58). The volume of the centrol cavity of GroEL also doubles by these conformational changes. At the same time, a bound SP is released and encapsulated by the cavity (57). The structural transition initiated by the binding of nucleotide also enables the binding of GroES to the former substrate binding sites. A $GroEL_{14}/GroES_7/ATP_7$ *cis* complex is thus formed. This $GroEL_{14}/GroES_7/ATP_7$ *cis* complex is a folding active species in the GroE cycle (20). The binding of GroES to $GroEL/ATP_7$ occurs very rapidly (>4 x 10^7 $M^{-1}s^{-1}$) after the ATP-induced conformational change (7).However, the association of GroES to a $GroEL/ADP_7$ is slower (1 x 10^5 $M^{-1}s^{-1}$) (27). During this rearrangement the walls of the cavity change their character from hydrophobic to hydrophilic, thus giving polypeptides the chance to fold without intermolecular interactions (57). Binding of nucleotide and GroES to one ring weakens the binding of ATP and GroES to the opposite ring, but it does not affect the binding of a SP to the *trans* ring (58).

Unlike the major conformational changes occurring in GroEL, the binding of GroES depends on the twisting of the apical domain relative to the equatorial domain (48). The number of GroES mobile loops interacting with GroEL in the binding sites has been studied using a fused 7-mer of GroEL. The experimental results indicated that GroEL was capable of accommodating as many as 4 mutant subunits in the heptametric ring without diminishing the yield of GroEL-GroES complex (50). Although SPs and

GroES share common binding sites, it seems that SPs only binds to a subset of the 7 available sites, which leaves the remaining sites available for interaction with the mobile loops of GroES (49). As a result of conformational changes, GroES replaces SPs in the binding sites. The binding of GroES to GroEL also enlarges the volume of the central cavity to about 170,000 $Å^3$, approximately twofold. This is the ultimate limit to the size of proteins that can be accommodated. Using multimers of green fluorescent protein, together with theoretical considerations of packing density, it has been calculated that the upper limit is approximately 58,000 dalton (49). Upon the binding of GroES to GroEL the polarity of the surface of the central cavity takes a dramatic change (49). In previous state the surface was hydrophobic. In contrast, in the GroES binding state the surface changes to hydrophilic. It remains in this hydrophilic state in the rest of the reaction cycle. The switch between hydrophobic and hydrophilic states and their duration is sensed by the SP.

1.5.3. ATP hydrolysis and polypeptide releasing

It is believed that all seven sites are associated with ATP at the *in vivo* ATP concentrations (50). Fluorescence anisotropy measurements showed that following binding of GroES and ATP to a GroEL-polypeptide binary complex to form a *cis* ATP complex, SP is released into the central channel within a second (43). ATP hydrolysis at a rate of 0.12 s^{-1}, occurs in the asymmetric $GroEL_{14}/GroES_7/ATP_7$, triggering the ring for subsequent steps in the cycle (43). The ATP hydrolysis is potassium dependent (50). While inorganic phosphate is released from the active site, ADP is locked in the active site, leading to the formation of a $GroEL_{14}/GroES_7/ADP_7$ complex (11). Polypeptide folding is believed to occur during this time and after formation of the *cis* ADP. SPs only

have about 6s to fold into the correct conformation prior to disruption of the folding-active chaperonin, estimated by the schedule of events on GroEL dictated by ATP binding and hydrolysis (47). Only a portion of SPs reach their native state during this period. The remaining nonnative SPs either go into another round GroE cycle or are degraded by proteases. It is critical for cells to remove damaged proteins, preventing the clogging of the chaperone machinery (47).

1.5.4. GroES and ADP release

The binding of ATP to the opposite GroEL ring induces the release of bound ADP and GroES from the *cis* ring (14). The signal appears to be transferred via the equatorial domains. The binding of another substrate polypeptide to the *trans* ring only occurs after ATP hydrolysis in the *cis* ring (49). The binding of nonnative polypeptide to the *trans* ring enhances the rate of the ATP-dependent ligand release 20-50 fold (43). After GroES leaves, polypeptide is released. During this process, the volume of the central cavity contracts from 175,000 $Å^3$ to 85,000 $Å^3$ (49). The hydrophobic surface of the central cavity is reinstalled. The ADP is released from the active site with the contraction of the intermediate domain. The rate limit in the GroE ATPase cycle is likely set by the rigid body movements in the GroE complexes, which are induced by ATP binding and transferred to the other ring via the equatorial domains (20). The apparent rate for the whole process is 0.042 s^{-1} in the absence of SP and about 0.6 s^{-1} in the presence of SP.

1.6 Allosteric states change

The allostery consideration of GroEL is described by a model of nested cooperativity (figure 1-5) (58).

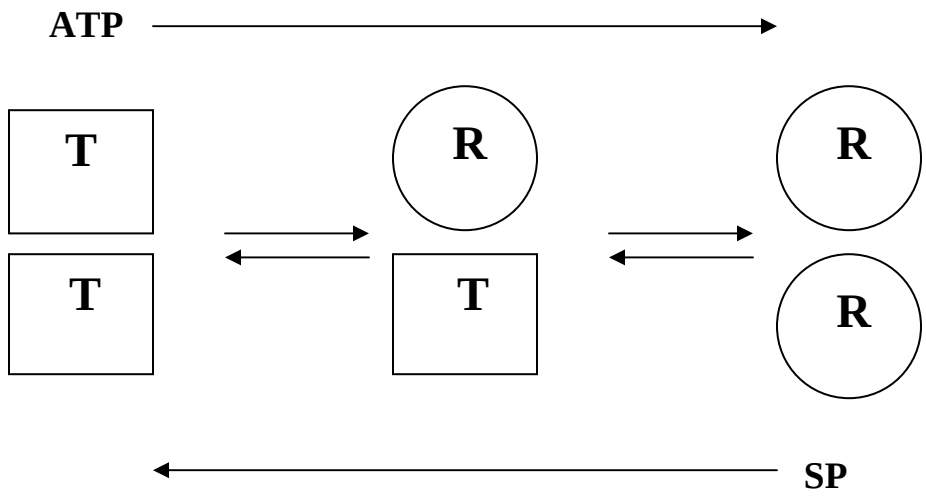


Figure 1-5. GroEL allosteric states change promoted by ATP and unfolded SP. ATP binding to a ring promotes the transition shift from T state to R state, following positive cooperativity in that ring. ATP can not bind to the second ring unless all 7 ATPs are hydrolyzed in the first ring, following a negative cooperativity in that ring. (Figure from Yifrach & Horovitz 1995).

Before ATP binding, a single ring of GroEL is in the tight (T) state with low affinity for ATP and high affinity for unfolded SP. ATP binds to the single ring at low concentration (<100μM) results in a transition to relaxed (R) state (TT to TR) with observed positive cooperativity between subunits of a ring. The negative allostery exists between two rings. Because of this inter-ring negative cooperativity, the transition of the TR to RR only exists at relatively higher concentration of ATP (59). In the presence of GroES, a third allosteric state, R' state, occurs. GroES binding to the *cis* ring results in a large volume increase of the central cavity, forming the "bullet" complex. The increasing volume of central cavity provides increased hydrophilicity which favors to the SP folding. GroES "locks" the nucleotide in the *cis* ring. The nucleotide can not be released unless ATP binds to the *trans* ring causing the *cis* ring complex to dissociate. Besides

ATP, it is believed that magnesium ion (Mg^{2+}), potassium ion (K^{+}) also have allosteric effects on GroEL (51).

1.7 Study goal

The purpose of this thesis is to develop a method to monitor the GroEL structure change in the presence of SP, ATP and GroES. Since FRET monitors the distance change between donor and acceptor, the structural change of GroEL may be monitored because of the rigid body motions it undergoes. Therefore, I created GroEL mutants which can be labeled with fluorescent probes that can be monitored for GroEL allosteric transition.

Chapter 2

Materials and Equipment

2.1 Materials

The plasmid pGEL1(cloned GroEL wild type gene) is a gift from Dr. Edward Eisenstein's lab. The host strains for plasmids for cloned GroEL mutations gene here are E. coli XL-I blue super competent cells. The competent cells for GroEL mutants protein expression are BL 21 competent cells.

The other materials are listed in Table 2-1.

Table 2-1. Material list.

Material	**Manufacturer/Catalog Number**
QIAprep spin miniprep kit(50)	Qiagen cal.no.27104
Quikchange site-directed mutagenesis kit	Stratagene cal.no.200518
Primers	MWG biotech
Restriction enzymes: *Fsp I* *PvuII* *NaeI* *NsiI* *BseYI* *NcoI* *Bln I* *Bsm I* *Stu I*	New England Biolabs cat.no.R0135S New England Biolabs cat.no.R0151S New England Biolabs cat.no.R0190S New England Biolabs cat.no.R0127S New England Biolabs cat.no.R0635S New England Biolabs cat.no.R0193S Roche cat.no.1558161 New England Biolabs cat.no.R0134S New England Biolabs cat.no.R0600S
AMP(Ampicillin)	Sigma cat.no.A9518
IPTG(Isopropyl B-D-Thiogalactopyranoside)	Sigma cat.no.I5502
Tris	FisherScientic cat.no.BP154
EDTA(Ethylenediaminetetraacetic acid disodium salt)	FisherScientic cat.no.E458
DTT (DL-Dithiothreitol)	Sigma cat.no D0632
Strep sulfate(Strepomycin sulfate)	Sigma cat.no.S9137
Protease inhibitor	Roche Diagnostics GmbH cat.no.11697498001
DEAE Sepharose^{TM} Fast Flow	Amersham Biosciences cat.no.17- 0709-01
Sephacryl S-300 HR	Amersham Biosciences cat.no.17- 0599-01
CM Sepharose^{TM} Fast Flow	Amersham Biosciences cat.no.17-0719-10
Sepharose^{TM} G-50 Fine	Amersham pharmacia Biotech AB cat.no.17-0042-07
Ammonium sulfate	Sigma cat.no.A6387
Bradford reagent	Sigma cat.no B6916
Acetone	J.T.Baker cat.no.9006-06
Centriplus YM-50	Millipore cat.no.4423

Centriplus YM-10	Millipore cat.no. 4421
Centricon YM-50	Millipore cat.no.4424
Microcon YM-50	Millipore cat.no.42416
PD-10 column	Amersham Biosciences cat.no.17- 0815-015
Gdn-HCl(Guanidine Hydrochloride)	Sigma cat.no.G4505
Streptavidin - Cy3	Invitrogen cat.no.43-4315
Streptavidin - Cy5	Invitrogen cat.no.43-4316
F5M(fluorescein-5-maleimide)	Invitrogen cat.no.F150
T5M(tetramethylrhodamine-5-maleimide)	Invitrogen cat.no.T6027
488(Alexa Fluor 488 C5-maleimide)	Invitrogen cat.no.A10254
546(Alexa Fluor 546 C5-maleimide)	Invitrogen cat.no.A10258
DMSO(Dimethyl Sulfoxide)	FisherScientic cat.no.D128
ATP(Adenosine 5'-triphosphate)	Sigma cat.no.A7699
ADP(Adenosine 5'-diphosphate)	Sigma cat.no.A2754
Hexokinase	Sigma cat.no. H4502
BSA(Bovine serum albumin)	Bio-Rad cat.no.500-0007
TECP(Tris (2-carboxyethyl) phosphine hydrochloride)	Sigma cat.no.C4706
Glycerol	Sigma cat.no.G8207

2.2 Equipment

The main equipment used in this thesis are listed Table 2-2. Cary 100 UV-Vis spectrophotometer was used to measure GroEL or GroES monomer and dye concentrations. Fluorescence spectrometer LS50B was used FRET and quenching fluorescent probes experiments. SDS-PAGE gels were scanned by Personal Densitometer SI and Agarose gels by Storm 860.

Table 2-2. Equipment list.

Equipment	**Software**	**Manufacturer**
Cary 100 UV-Vis spectrophotometer	Cary WinUV Software	Varian
Fluorescence spectrometer LS50B	FL WinLab software	Perkin Elmer
Personal Densitometer SI	PDSI, ImageQuant5.1 software	Molecular Dynamics, Inc
Storm 860	Storm Scanner control software	Molecular Dynamics, Inc

Chapter 3

General methods

3.1 Site-directed mutagenesis for GroEL and GroEL(sr) mutants

In this thesis, GroEL mutants came from three different backgrounds: 1) wild type (GroEL(wt)); 2) Cys-free background (GroEL(cf)); 3) single ring GroEL (GroEL(sr)). To illustrate the relationship among the GroEL wild type (or GroEL(sr)) plasmid and the mutants plasmids, a flow chart (Figure 3-1) is presented to simplify the description. For example, to obtain GroEL(wt)$_{K242C/N527C}$ mutant, GroEL(wt)$_{K242C}$ plasmid was used as the template for PCR and so on.

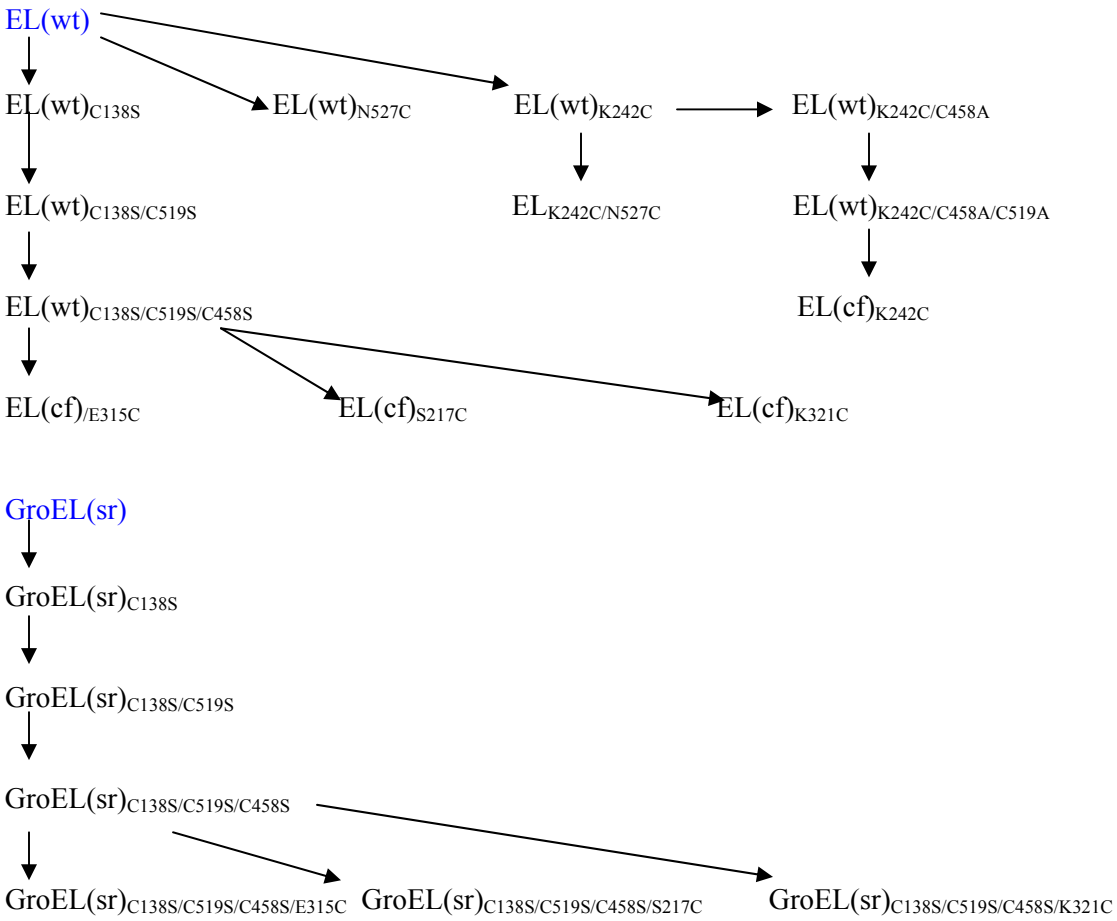


Figure 3-1. Relationships among GroEL(wt) plasmid and its mutants plasmids (top) and GroEL(sr) plasmid and its mutants plasmids (bottom).

A table (Table 3-1) is provided to describe each mutant with a template and a pair of PCR corresponding primers to create each GroEL (or GroEL(sr)) mutant. To gauge the

success of each mutagenesis, a restriction enzyme was designed for each corresponding mutant (Table 3-1).

Table 3-1. Information about template, primers and restriction enzyme for each mutagenesis.

Mutant	Template	Primer*	Restriction enzyme
EL(wt)$_{K242C}$	EL(wt)	K242C-s: CTGGAAGCTGTTGCCTGC GCAGGCAAACCGCTG; K242C-ns: CAGCGGTTTGCCTGCGCA GGCAACAGCTTCCAG.	*FspI*
EL(wt)$_{N527C}$	EL(wt)	N527C-s: CCGACCTGCCGAAATGCG ATGCAGCAGACTTAGGC; N527C-ns: GCCTAAGTCTGCTGCATC GCATTTCGGCAGGTCGG.	*PvuII*
EL(wt)$_{K242C/N527C}$	EL(wt)$_{K242C}$	N527C-s: CCGACCTGCCGAAATGCG ATGCAGCAGACTTAGGC; N527C-ns: GCCTAAGTCTGCTGCATC GCATTTCGGCAGGTCGG.	*PvuII*
EL(wt)$_{K242C/C458A}$	EL(wt)$_{K242C}$	C458A-s: GCGTCAGATCGTATTGAA CGCCGGCGAAGAACCG; C458A-ns: CGGTTCTCCGCCGGCGTT CAATACGATCTGACGC.	*NaeI*
EL(wt)$_{K242C/C458A/C519A}$	EL(wt)$_{K242C}$ /C458A	C519A-s: GATCACCACCGAAGCCAT GGTTACCGACCTGCCG; C519A-ns: CGGCAGGTCGGTAACCAT GGCTTCGGTGGTGATC.	*NsiI*
EL(cf)$_{K242C}$	EL(wt)$_{K242C}$ /C458A/C519A	C138S-s: GCGCTGTCCGTACCCAGC TCTGACTCTAAAGCG; C138S-ns: CGCTTTAGAGTCAGAGCT GGGTACGGACAGCGC.	*BseYI*
EL(wt)$_{C138S}$	EL(wt)	C138S-s: GCGCTGTCCGTACCCAGC TCTGACTCTAAAGCG; C138S-ns: CGCTTTAGAGTCAGAGCT GGGTACGGACAGCGC.	*BseYI*
EL(wt)$_{C138S/C519S}$	EL(wt)$_{C138S}$	C519S-s:	*Nco I*

		GATCACCACCGAATCCAT GGTTACCGACCTGCCG; C519S-ns: CGGCAGGTCGGTAACCAT GGATTCGGTGGTGATC.	
EL(cf)	EL(wt)$_{C138S/C519S}$	C458S-s: CCGCTGCGTCAGATCGTA TTGAATTCCGGCGAAGAA CCG; C458S-ns: CGGTTCTTCGCCGGAATT CAATACGATCTGACGCAG CGG.	*EcoRI*
EL(cf)$_{E315C}$	EL(cf)	E315C-s: GCTGGAAAAAGCAACCC TGTGCGACCTAGGTCAGG CTAAACG; E315C-ns: CGTTTAGCCTGACCTAGG TCGCACAGGGTTGCTTTT TCCAGC. (designed by Dr. John Grason)	*Bln I* (designed by Dr. John Grason)
EL(cf)$_{S217C}$	EL(cf)	S217C-s: GCAGTAGAACTGGAATG CCCGTTCATCCTGCTG; S217C-ns: CAGCAGGATGAACGGGC ATTCCAGTTCTACTGC.	*Bsm I*
EL(cf)$_{K321C}$	EL(cf)	K321C-s: CCTGGAAGACCTGGGTCA GGCCTGCCGTGTTGTGAT CAAC; K321C-ns: GTTGATCACAACACGGCA GGCCTGACCCAGGTCTTC CAGG.	*Stu I*
GroEL(sr)$_{C138S}$	GroEL(sr)	C138S-s: GCGCTGTCCGTACCCAGC TCTGACTCTAAAGCG; C138S-ns: CGCTTTAGAGTCAGAGCT GGGTACGGACAGCGC.	*BseYI*
GroEL(sr)$_{C138S/C519S}$	GroEL(sr)$_{C138S}$	C519S-s: GATCACCACCGAATCCAT GGTTACCGACCTGCCG; C519S-ns: CGGCAGGTCGGTAACCAT GGATTCGGTGGTGATC.	*Nco I*
GroEL(sr)$_{C138S/C519S/C458S}$	GroEL(sr)$_{C138S/C519S}$	GroEL(sr)C458S-s: GATCGTATTGAATTCCGG CGAGGCGCCGGCGG; GroEL(sr)C458S-ns:	*EcoRI*

		CCGCCGGCGCCTCGCCGGAATTCAATACGATC.	
GroEL(sr)$_{C138S/C519S/C458S/E315C}$	GroEL(sr)$_{C138S/C519S/C458S}$	E315C-s: GCTGGAAAAAGCAACCCTGTGCGACCTAGGTCAGGCTAAACG; E315C-ns: CGTTTAGCCTGACCTAGGTCGCACAGGGTTGCTTTTTCCAGC. (designed by Dr. John Grason)	*Bln I* (designed by Dr. John Grason)
GroEL(sr)$_{C138S/C519S/C458S/S217C}$	GroEL(sr)$_{C138S/C519S/C458S}$	S217C-s: GCAGTAGAACTGGAATGCCCGTTCATCCTGCTG; S217C-ns: CAGCAGGATGAACGGGCATTCCAGTTCTACTGC.	*Bsm I*
GroEL(sr)$_{C138S/C519S/C458S/K321C}$	GroEL(sr)$_{C138S/C519S/C458S}$	K321C-s: CCTGGAAGACCTGGGTCAGGCCTGCCGTGTTGTGATCAAC; K321C-ns: GTTGATCACAACACGGCAGGCCTGACCCAGGTCTTCCAGG.	*Stu I*

***Red*: changed nucleotide for changing the individual amino acid into Cys; green: changed nucleotide for creating or deleting a restriction enzyme splice site; blue: changed nucleotide for changing the individual amino acid into Cys and also creating or deleting a restriction enzyme splice site.**

Since the protocols for all mutagenesis of GroEL mutants are the same except for using different primers and restriction enzymes, here I only describe the procedure for the first GroEL mutant (EL(wt)$_{K242C}$):

By using Stratagene Quick-Change Kit, EL(wt)$_{K242C}$ mutant was introduced into EL(wt) plasmid. The primers were K242C-s and K242C-ns (see Table 3-1). The PCR reaction mixture included 125ng of each primer, 20ng EL(wt) plasmid, reaction buffer, dNTP mix, Pfu Turbo DNA polymerase. PCR cycling followed 3 programs:

1. 95°C, 30 seconds, 1 cycle.

2. 95°C, 30 seconds,

   55°C, 1 minute,

   68°C, 13 minutes,

   repeat 16 cycles.

3. 68°C, 10 minutes, 1 cycle.

The PCR product was treated with Dpn I enzyme for 1 hour at 37°C to cut the EL(wt) plasmid. The Dpn I-treated plasmid was transferred in XL-I blue supercompetent cells by heat shock, then streaked on a LB+AMP plate and incubated overnight at 37°C. The next day several colonies were inoculated into 25 ml LB+AMP medium and incubated overnight at 37°C. The procedure for extracting $EL(wt)_{K242C}$ mutant plasmid was provided by the QIAprep Spin Miniprep Kit. To check the success of mutagenesis a restriction enzyme (FspI) was used. 1 µg of plasmid, 0.5 µl of FspI, a final concentration of 1X reaction buffer and autoclaved distilled water were mixed in total volume of 13 µl, following with incubation about one hour at 37°C water-bath. The mixture with 2µl 5X agarose dye was loaded on a 0.7% agarose gel. The mutagenesis result also was confirmed by DNA sequencing (from the University of Maryland DNA sequencing facility). Since XL-I blue supercompetent cells are the ideal cells for storing DNA, but not the best cells for protein expression, the mutant plasmid was transferred into the BL 21 competent cells. The glycerol stock for the mutant was made with 0.7 ml culture and 0.3 ml autoclaved glycerol, then stored at -80°C for future using.

3.2 Purification for GroEL(wt)

GroEL(wt) glycerol stock was streaked on a LB+Amp plate and incubated at 37°C overnight. Three single colonies were inoculated into three 1.5L LB media containing

100μg/μl Amp and incubated at 37°C until the OD reached at about 0.6 at 595nm. A final concentration of 0.5 mM IPTG was added to overexpress GroEL. The culture was incubated at 30°C for 16 hours. Cells were harvested by centrifugation and resuspended in buffer containing 50mM Tris, pH8, 5mM $MgCl_2$, 1mM EDTA, 1mM DTT and 3 tablets of protease inhibitor. The cells were lysed in 50 ml portions by a sonicator set to power level 5, 50% duty cycle, for 75 seconds. The cell debris were removed by centrifugation. A final concentration of 8 mg/ml streptomycin sulfate was added to remove nucleic acid. Removal of precipitate was done by centrifugation. The supernatant was loaded on a 500 ml DEAE Sepharose Fast Flow column which was pre-equilibrated with 400 ml of 200mM Tris pH 8, then in 1800 ml Buffer A (50 mM Tris pH 8, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT). Following elution of flow-through, the protein was eluted over 2 L gradient from 0 mM to 50 mM NaCl on a FPLC system. The GroEL fractions were determined by SDS-PAGE and pooled. To concentrate GroEL, saturated ammonium sulfate was added to a final concentration of 65%. The solution was stored at 4°C overnight while slowly stirring. The solution was centrifuged to obtain the precipitate. S300 buffer containing 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1mM EDTA, 1 mM DTT was used to resuspend the precipitate. The solution was loaded onto a 300 ml S300 Sephacryl gel filtration column which was pre-equilibrated with 300 ml S300 buffer. The flow-through was pooled and concentrated above 10 mg/ml measured by Bradford. The protein was stored at -80°C until further purifying steps were needed.

In our lab, acetone treatment (communicated by Mark T. Fisher) is a very important step to obtain high purity of GroEL. The solution is brought to a final concentration of 45% of acetone, added dropwise to the protein solution while stirring. The solution was

centrifuged and the precipitate was resuspend with a similar volume of 10:10 buffer (10 mM Tris pH 7.5, 10 mM $MgCl_2$). Only GroEL was resuspend in this buffer. The contaminating proteins remained cloudy and were removed by a second centrifugation. A final concentration of 65% saturated ammonium sulfate was added to the supernatant. The pellets were resuspended in 10:10 buffer. A PD-10 column was used for de-salting. The final product was concentrated using an YM-50. Since GroEL concentration was checked by absorbance at 280 nm using 300µl of 8 M Gdn-HCl which dissociated GroEL 14 mers into GroEL monomer, 80µl distilled water and 20µl of protein sample. The dilution factor (300+20+80)/20 (=20) and an extinction coefficient (ε) of 9600 $M^{-1}cm^{-1}$ were used for calculation. In this experiment, a cuvette with 1 cm path length (b) was used. From the equation A= ε*b*c, concentration (c) equals to A/(b*ε). For example, if the absorbance is 0.5, then the GroEL monomer concentration will be 0.5*20/9600/1 (=1.04 X 10^{-3}) M.

The purity of GroEL(wt) was confirmed by tryptophan fluorescence measurement. GroEL does not have native tryptophan residues, but a typical 40 KDa protein has about 4 Trp residues. Measuring the trp fluorescence emission at 350 nm by excitation at 295 nm indicates the presence of contaminating proteins. Since BSA has 4 trp residues/molecule, it was diluted into 8M Gdn-HCl to create a standard curve for the contaminating proteins. The lower the Trp fluorescence emission, the higher is the GroEL(wt) purity. The ratio of the moles of the contaminating proteins to the moles of GroEL(wt) 7 mer represents the ratio of GroEL(wt) rings occupied by SPs. A preparation of GroEL was judged acceptable if more than 85% of the heptametrical rings are free of contaminating proteins.

3.3 Purification for GroEL mutants

The procedure for purifying GroEL mutant was the same for EL(wt) except using lower concentration of DTT for GroEL(wt) and higher concentration of DTT for GroEL mutants.

3.4 Purification for GroES(wt)

The purification protocol used in our lab was modified from a published paper (13). The plasmid for GroES is pGES1. The procedure for obtaining GroES lysate was the same as GroEL. The lysate was treated at 70°C-74°C for 10 minutes to remove contaminating proteins. The solution was centrifuged to remove the precipitate. A final concentration of 65% of saturated ammonium sulfate was added to the supernatant. The mixture was stored at 4°C overnight while slowly stirring. In the next day, the solution was centrifuged. The pellet was resuspended in 20 ml of G50 buffer containing 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM DTT. The sample was loaded onto a G50 column which was pre-equilibrated with 300 ml of G50 buffer. About 60 ml of GroES was collected. The pH was modified to around pH 5.05 by rapid addition of NaOAC, pH 5 and applied to a 75 ml SP Sepharose HP column pre-equilibrated with 400 ml SP buffer (50 mM NaOAC pH 5, 0.1 mM EDTA, 1 mM DTT). For each running, about 50 ml of sample was loaded onto the column. GroES was eluted about 750 ml using a 0 mM-200 mM NaCl gradient. GroES fractions were determined by SDS-PAGE gel. The GroES fractions from three runs were pooled together and precipitated by saturated ammonium sulfate to a final concentration of 65%. The solution was stored at 4°C overnight while stirring. The ammonium sulfate precipitate was centrifuged and the pellet was resuspended in 4.8 ml of 10 mM Tris, pH7.5. The material was desalted by a PD-10

column pre-equilibrated by 25 ml of the same solution. The final product was concentrated in an YM-10. The absorbance at 280 nm with an extinction coefficient of 1200 $M^{-1}cm^{-1}$ was used to determine the GroES monomer concentration.

3.5 Labeled methods

In this thesis, all GroEL mutants are cysteine (Cys) mutants with free thiols. Because maleimides are outstanding chemicals to form thiol-selective modification, the thiol of a Cys residue can react with the double bond of the maleimide to produce a thioether (Figure 3-1).

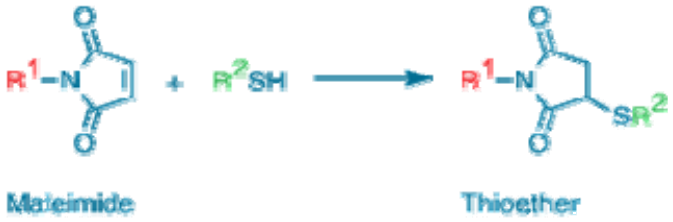


Figure 3-2 Chemical reaction of a Cys residue with a maleimide. (figure from Invitrogen website).

Several maleimides acted as donor and acceptor for this thesis are listed following (Table 3-2):

Table 3-2 Some properties of the donors and acceptors used in this thesis.

Maleimide	Probe Name	Abs max. (nm)	Em max. (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Förster Distance R_0 (Å)
Donor Acceptor	Fluorescein(F5M) Tetramethylrhodamine(TMR)	492 541	515 567	83,000 95,000	54
Donor Acceptor	Cy3 Cy5	548 646	562 664	150,000 250,000	53
Donor Acceptor	Alexa fluor 488 Alexa fluor 546	493 554	516 570	72,000 93,000	64

3.5.1 GroEL mutants labeled with donor and acceptor separately

All the following operations were performed in dim light to minimize photo bleaching. Stock solution of each probe was prepared by dissolving a granule of the

probe powder into 50 or 100 µl of fresh DMSO. 5 µl of solution was used to measure the probe concentration by absorbance using an appropriate extinction coefficient.

The labeling reaction could be done in three different methods:

1) **Method B**: At the beginning of the reaction, 150 or 120 µM fluorescent maleimide (F5M, TMR, Cy3, Cy5, Alexa fluor 488 or Alexa fluor 546) was added to 25 or 20µM (monomer concentration) of the GroEL mutant of interest in 10:10 buffer(10mM, 10mM MgAc), (pH7.2 for F5M and TMR; pH 7 for Alexa fluor 488 or Alexa fluor 546) for 90 min with 900 rpm shaking at 37°C. The mixture was brought to 10 mM of DTT to quench the reaction. The free dyes were removed by ultrafiltration and PD-10 gel filtration column which was pre-equilibrated with 10:10 buffer. The labeled dye concentration was estimated from the maximum absorbance of each probe. The labeled GroEL protein concentration was checked by comparing it with a standard curve of a known concentration of GroEL on a SDS-PAGE gel.

2) **Method B':** the difference between **Method B** and **Method B'** is in the beginning of reaction, just the same concentration of fluorescent maleimide as the protein concentration was added to the mixture, then after 15 minutes of incubation, the same concentration of dye was added again and repeated this addition 4 times. The other parts of the produce is the exact same as **Method B**.

3)**Method C**: in this method, the reaction buffer was 50 mM Tris, pH7.5, 50mM KCl, 0.2mM TCEP, 20% glycerol. Excess of dye was added to 20 µM of the mutant GroEL. The labeling reaction was performed at 4° for about 24 hours. After this, the procedure was the same as **Method B**.

3.5.2 GroEL mutants or GroEL(wt) labeled with donor following by labeling with acceptor

There were also three different ways to label GroEL with donor following by labeling with acceptor:

1) **Method DA**: label GroEL protein with donor firstly by **Method B,** then label protein with acceptor with **Method B** again.

2) **Method D'A'**: label GroEL protein with donor firstly by **Method B',** then label protein with acceptor with **Method B'** again.

3) **Method CC**: use **Method C** to label GroEL protein with donor following with labeling acceptor with **Method C**.

3.5.3 GroEL mutants labeled with acceptor following by labeling with donor

GroEL mutants were labeled with acceptor firstly by Method B', then labeled with donor by method B' again (called **Method A'D'**).

3.5.4 Co-mingling for GroEL mutant labeled with donor only and GroEL mutant labeled with acceptor only

This procedure is called "co-mingling" from a protocol developed by Sarah C. Wehri(56) (Figure 3-3).

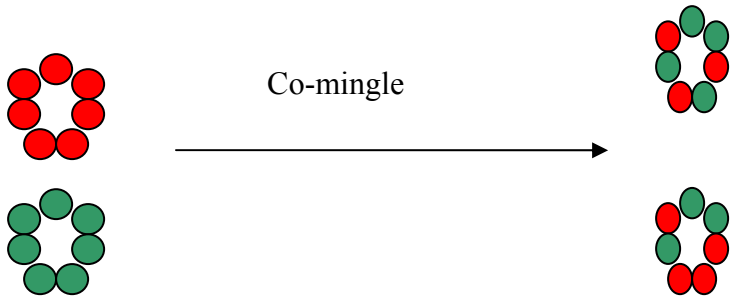


Figure 3-3. Statistical re-assembly to give an ensemble of GroEL 14 mers. Red: GroEL subunits labeled with donor; green: GroEL subunits labeled with acceptor. (Figure from Sarah C. Wehri).

A total final concentration of 20 μM of GroEL mutant of interest (GroEL mutant labeled with donor and GroEL mutant labeled with acceptor) was added to 10:10 buffer (pH7.2, with 5 mM DTT) in the ratio of interest. In these cases, the ratio of the labeled donor concentration to the labeled acceptor concentration was 1:1 or 1:2. A final concentration of 20% acetone was added to the GroEL mutant solution drop wise. The solution was mixed completely and left shaking at 900 rpm, room temperature for 10 minutes. This causes the dissociation of GroEL to monomers (Sarah C. Wehri, personal communication). Then the solution was brought to a final concentration of 65% ammonium sulfate to remove acetone, mixed thoroughly and left shaking at 900 rpm, room temperature for 30 minutes. The mixture was centrifuged in a biofuge centrifuge at 10,000 xg, room temperature for 20 minutes. The supernatant was discarded and the pellet was resuspended in 10:10 buffer (pH 7.2, with 5 mM DTT). To remove contaminating ammonium sulfate, several rounds of concentration/dilution were used with an appropriately sized centrifuge filter (like a centricon YM-50). The GroEL final concentration was checked by comparing it with a known concentration of GroEL on a SDS/PAGE gel.

3.6 FRET experiment

For FRET experiments, the excitation wavelength was 490nm or 460 nm for F5M + TMR pair; 514 nm for Cy3 + Cy5 pair; or 460 nm for Alexa fluor 488 + Alexa fluor 546 pair. A final monomer concentration of 0.2 μM, 0.4 μM or 1 uM of labeled GroEL protein was dissolved in FRET buffer 1 (10 mM Tris, pH 7.2; 10 mM MgAc, pH7.2; 10mM KAc, pH 7.2; 1mM DTT) or FRET buffer 2 (50 mM Tris, pH 7.5; 50 mM KCl; 10 mM$MgCl_2$; 1 mM DTT) to a total volume of 200 μl. The mixture containing the

labeled protein and the FRET buffer was called “protein only” group. 1-2 µl of a final concentration of 20 µM or 50 µM ATP was added into the same cuvette of the “protein only” group, the mixture then was called “protein+ATP” group. For “protein+ADP” group, 1-2 µl 60 µM ADP was added to the “protein only” group which already had 100 mM glucose and 0.01 unit/µl hexokinase. For some later experiments, ADP was treated with glucose and hexokinase to remove the contaminating ATP prior to using. Eventually, a final monomer concentration of 5 times as GroEL concentration of GroES(wt) was added the same cuvette of the “protein+ATP” or “protein+ADP” group, named “protein+ATP/ES” or “protein+ADP/ES” group. Spectra were taken for each of those groups.

Chapter 4

Example of mutagenesis result

Since the mutagenesis results for all the GroEL mutants (18 mutants) are repetitive, only one of them (GroEL(cf)$_{K242C}$ mutant) is described here. The other (17 mutants) are included in the appendix.

GroEL(cf)$_{K242C}$ mutant: The cloning of mutant GroEL(cf)$_{K242C}$ was checked by restriction enzyme (*BseYI*) digest (Figure 4). The agarose gel displayed an additional band for some GroEL(cf)$_{K242C}$ samples as compared to the GroEL(wt) plasmid. This result matched with the primers' design which created one more *BseYI* splice site. The mutagenesis result of GroEL(cf)$_{K242C}$ mutants was confirmed by DNA sequencing *(University of Maryland DNA sequencing facility).*

	1	2	3	4	5	6	7
Bse YI		-	+	-	+	-	+

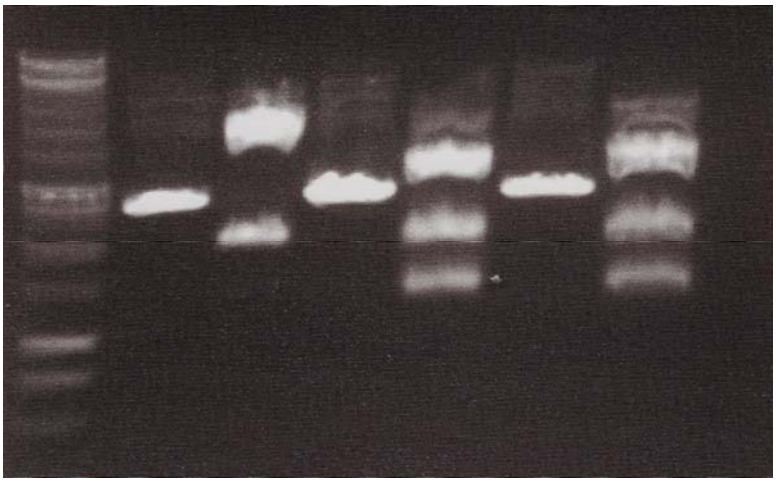


Figure 4. Result for mutagenesis of GroEL(cf)$_{K242C}$ digested with *BseYI*. (From left to right) Lane 1: 1kb DNA marker. Lanes 2 and 3: GroEL(wt) samples. Lanes 4-7: GroEL(cf)$_{K242C}$ samples. Lanes 2, 4, and 6: treated with *Bse YI*, and lanes 3, 5 and 7: treated with *Bse YI*.

Chapter 5

FRET results and Discussion

5.1 Introduction of FRET

5.1.1 The equation of FRET efficiency

FRET is a process by which an excited donor (D) is quenched by a nearby acceptor (A). FRET efficiency (E) is described by the equation

$$\mathbf{E=1/(1+(r/R_0)^6)}$$

in which R_0 is the Förster distance and r is the distance between D and A. Since FRET is a distance-dependent process, it should be possible to monitor the change in distance through the GroE cycle based on the change in donor and acceptor fluorescence (30).

5.1.2 Theoretical FRET efficiencies of F5M and TMR, Cy3 and Cy5, Alexa fluor 488 and Alexa fluor 546

In this thesis, three pairs of dyes were used : F5M and TMR, Cy3 and Cy5, Alexa fluor 488 and Alexa fluor 546.

A favorable FRET pair has following properties (30):

1) A high donor quantum yield in the absence of acceptor.

2) Overlap (*J*) of the donor emission and acceptor absorbance spectra. The overlap spectra of fluorescent probes (F5M and TMR, Cy3 and Cy5, Alexa fluor 488 and Alexa fluor 546) are shown in Figure 5-1.

3) ε, the extinction coefficient of a donor or acceptor, typically in units of $M^{-1}cm^{-1}$.

4) κ^2, a freely rotating set of fluorophores.

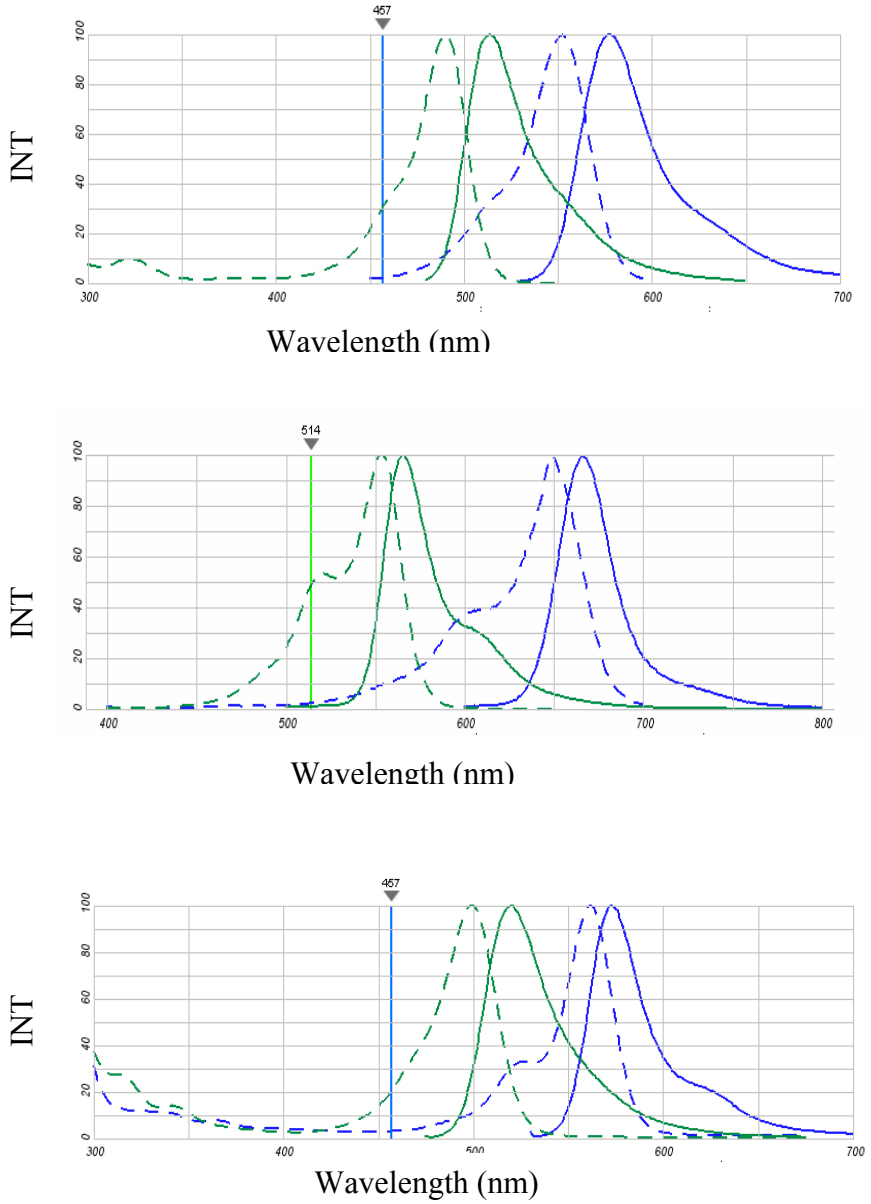


Figure 5-1. Absorption (dotted line) and fluorescence emission (solid line) of three pairs of probes. *Top*: F5M (green) and TMR (blue) excited at 457 nm. Middle: Cy3 (green) and Cy5 (blue) excited at 514 nm. *Bottom*: Alexa fluor 488 (green) and Alexa fluor 546 (blue) excited at 514 nm. (Figure from Invitrogen website).

Once the Förster distance is determined, the expected energy transfer for a dye pair can be calculated using the equation in section 5.1.1. The distance-dependant expected energy transfer for each pair is shown in figure 5-2.

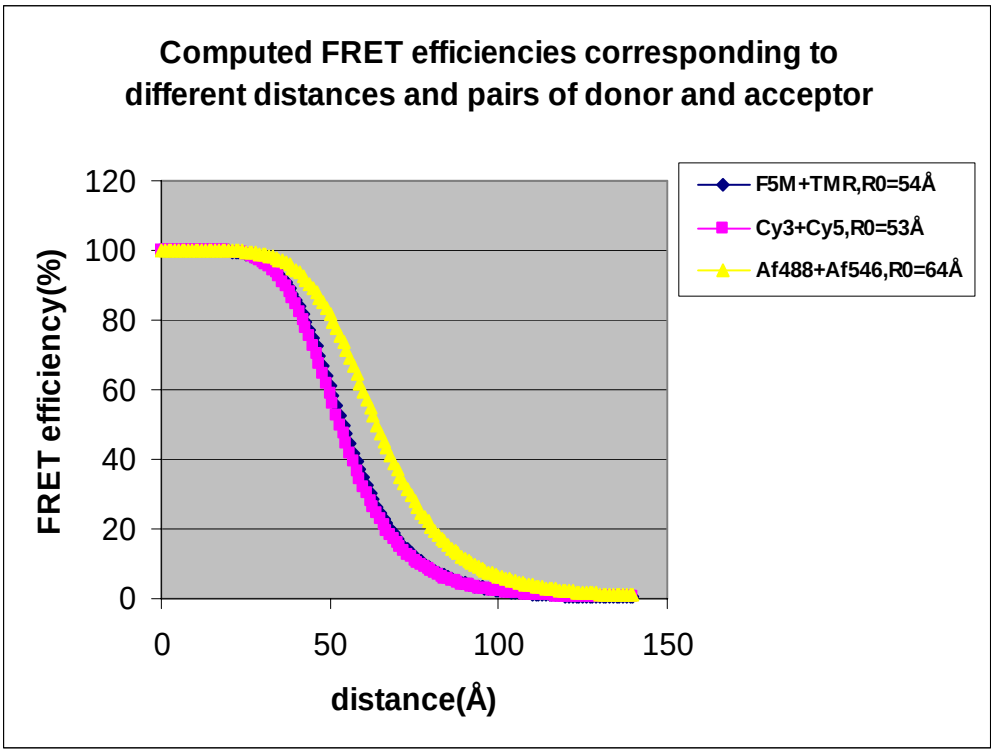


Figure 5-2. Computed FRET efficiencies for different distances between donor and acceptor and the three pairs: F5M and TMR; Cy3 and Cy5; Alexa fluor 488 and Alexa fluor 546.

5.2 Distance changes for adjacent and ""2-removed"" pairs on GroEL cis ring from T state to R and R' states

When a GroEL ring transitions from T to R and then R' state, the position of each residue on this ring changes because of the large conformational changes the ring undergoes. Thus the distance of the same residue between adjacent subunits or subunits "2-removed" from one another (Figure 5-3) may increase or decrease depending on the residue.

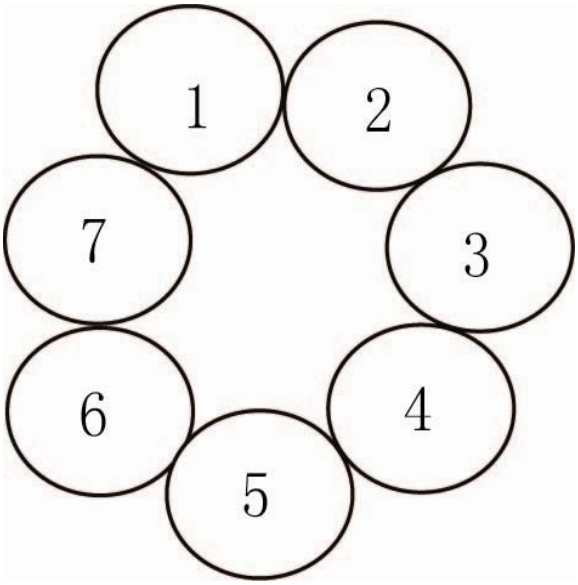
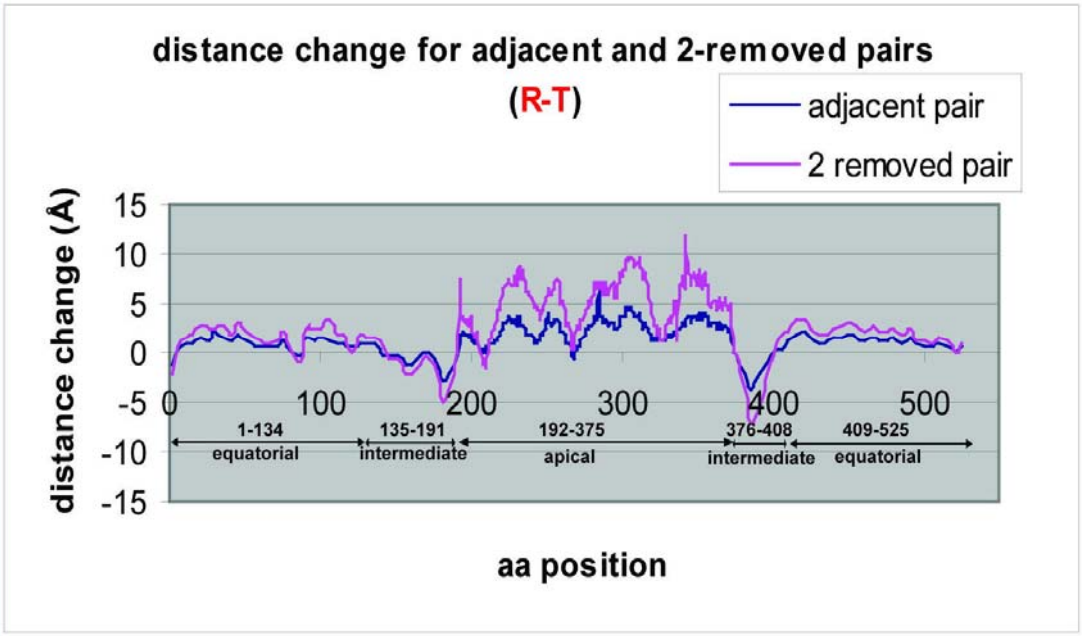


Figure 5-3. Scheme of adjacent subunits and "2-removed" subunits on a ring. Each circle represents a subunit. For example, subunits 1 and 2, and subunits 1 and 7 may be called adjacent subunits; subunits 1 and 3, and subunits 1 and 6 can be named as "2-removed" subunits, and so on.

The distance change for residues in the equatorial domain and intermediate domain is small, but can be large for residues in the apical domain. This reflects the fact that the equatorial and intermediate domains form a stable, relatively immobile platform, upon which the allosteric motions of the apical domain can be based (Figure 5-4). For this reason, a single residue mutation coupled with fluorescence can be used to probe the allosteric transitions of GroEL.



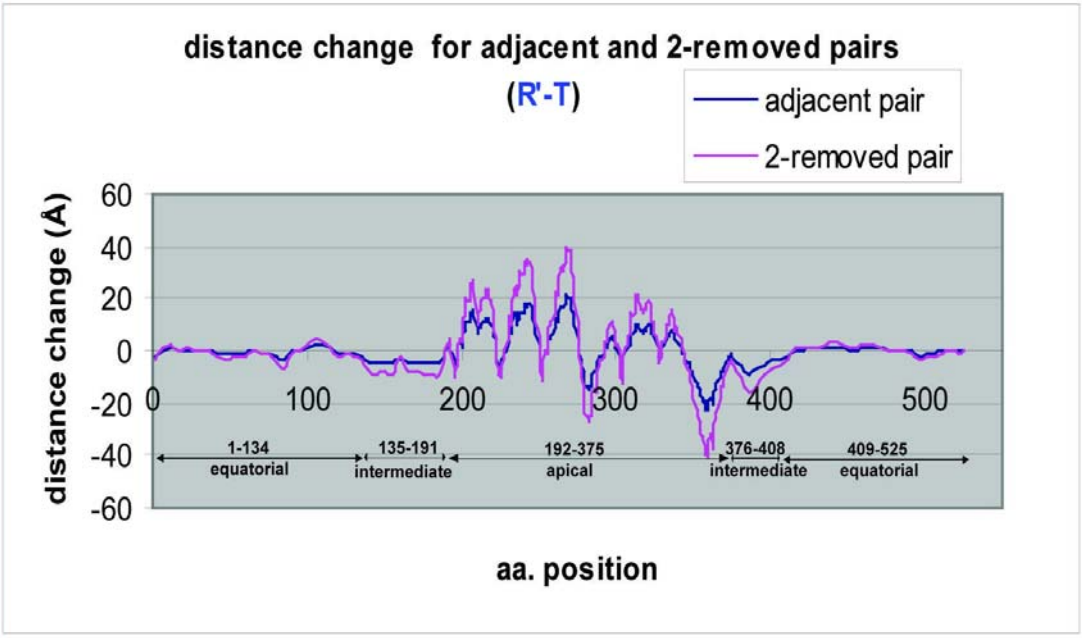


Figure 5-4. Distance change for GroEL cis ring residues on two adjacent or "2-removed" subunits. *Top*: from T to R state; *Bottom*: from T to R' state. The distance was measured from α–carbon of one residue to another with Rastop software.

5.3 GroEL(wt) may be labeled with fluorescent probes under some labeling condition

GroEL(wt), with three native Cys sites at 138, 458 and 519, was difficult to be labeled with F5M and TMR by Method CC at pH 7.5, obtained an average of 0.007 donor and 0.07 acceptor on each ring. FRET spectra showed that there was very little FRET occurring upon addition of ATP and ES (Figure 5-5). The observation matched with the conclusion previously obtained in our lab (21).

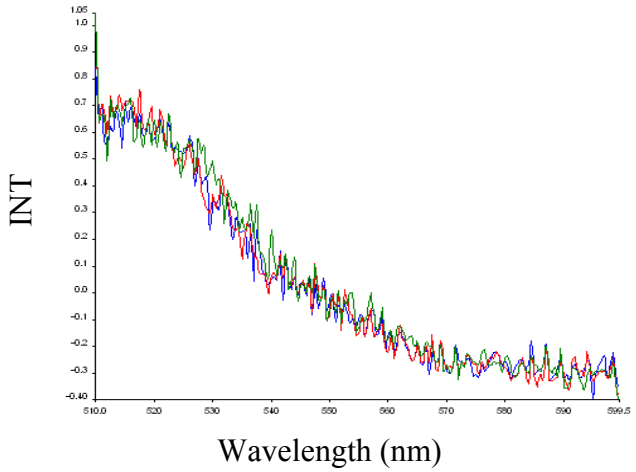


Figure 5-5. Original FRET spectra for GroEL(wt) labeled with F5M and TMR at pH7.5. Blue: "protein only" group; red:"protein + ATP" group; green: "protein + ATP/ES" group.

But GroEL(wt) was able to be labeled with that fluorescent pair by Method DA at pH 7.2, gained an average of 0.7 donor and 1.8 acceptors on each ring, and FRET could be observed (Figure 5-6). Since all the native Cys sites are in the equatorial domain, and the position of equatorial domain does not change a lot through the allosteric states, it contributed a significant background.

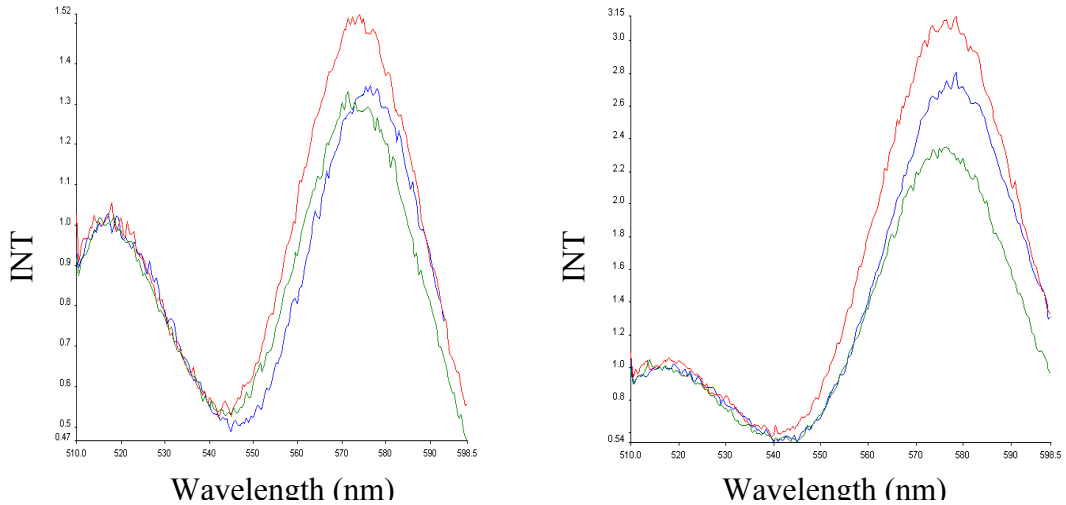


Figure 5-6. Normalized FRET spectra for GroEL(wt) labeled with F5M and TMR at pH7.2. *Left*: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. *Right*: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

5.4 GroEL(wt)$_{K242C/N527C}$ may not be the best mutant for monitoring the GroEL allosteric transition

When GroEL undergoes an allosteric transition from T state to R' state, the distance between residue 242 in the apical domain and residue 527 in the equatorial domain in the same subunit increases from 52Å to 82Å (Figure 5-7). When these residues are substituted with Cys, a fluorescent dye can be attached and the apical domain movement on the *cis* ring can be reported by FRET. This distance change is optimal for using of these fluorophores since it falls on the linear region of the expected energy transfer plot (Figure 5-2).

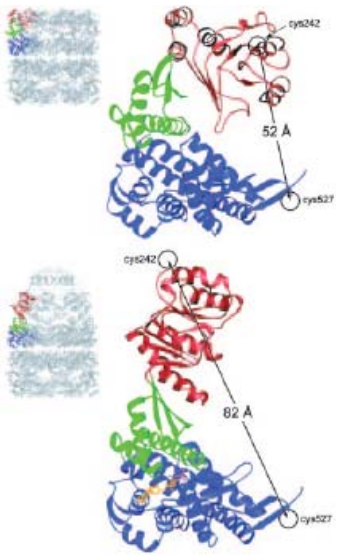


Figure 5-7. Positions of the two sites mutated to Cys. Red: apical domain, green: intermediate domain, and dark blue: equatorial domain. Top: T state. Bottom: R' state. (Motojima, et al, 2004).

The FRET results from GroEL(wt)$_{K242C/N527C}$ were unexpected. When labeled with F5M and TMR by Method CC, GroEL(wt)$_{K242C/N527C}$ was gained an average of 5.3 donors and 4.9 acceptors on each ring. FRET spectra showed that addition of ATP/ES and ADP/ES both increased E (Figure 5-8).

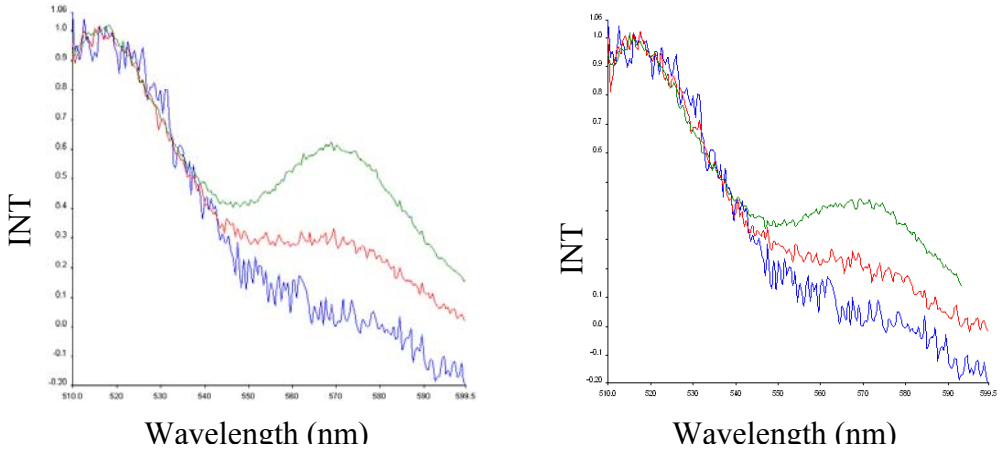


Figure 5-8. Normalized FRET spectra for GroEL(wt)$_{K242C/N527C}$ labeled with F5M and TMR. *Left*: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. *Right*: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

These results were totally opposite to what we expected. The cause of this may be that $GroEL_{K242C/N527C}$ has 5 Cys sites including 3 native sites and 2 introduced sites. When this mutant was obtained a high labeling extent, such as an average of 5.3 donors and 4.9 acceptors for each ring (it means 10.6 donors and 9.6 acceptors for a 14 mer), there were 3 kinds of effects which might be contributed to FRET: 1) intra-subunit effect including those pairs: 242-527 (which was the only one we wanted), 242-138, 242-458, 242-519, 527-138, 527-458, 527-519, 138-458, 138-519, 458-519; 2) inter-subunit; 3) inter-ring: *cis* ring-*trans* ring. Therefore the FRET results could not represent the transition change.

When $GroEL(wt)_{K242C/N527C}$ was labeled with Cy3 and Cy5, FRET spectra matched our expectation: addition of ATP alone and ATP/ES decreased E (Figure 5-9), consistent with an increase in the distance between residues of 242 and 527.

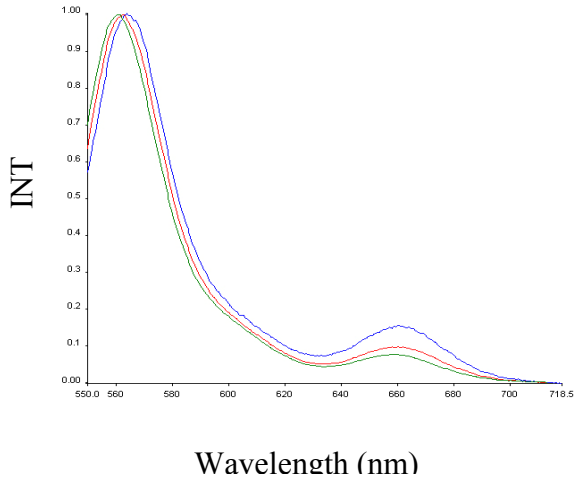


Figure 5-9. Normalized FRET spectra for $GroEL_{K242C/N527C}$ labeled with Cy3 and Cy5. Blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group.

5.5 No FRET occurs between individual GroEL molecules: shown by a simple mixing experiment of GroEL(wt)$_{K242C}$-F5M and GroEL(wt)$_{N527C}$-TMR

Use the simple mixture of GroEL(wt)$_{K242C}$-F5M and GroEL(wt)$_{N527C}$-TMR as our control experiment for the FRET system. In an aqueous environment, the distance between a residue in one GroEL$_{14}$ from another residue in another GroEL$_{14}$ is bigger than the distance over which FRET can occur. So no FRET should have occurred in the mixture. Our FRET results (Figure 5-10) confirmed our hypothesis.

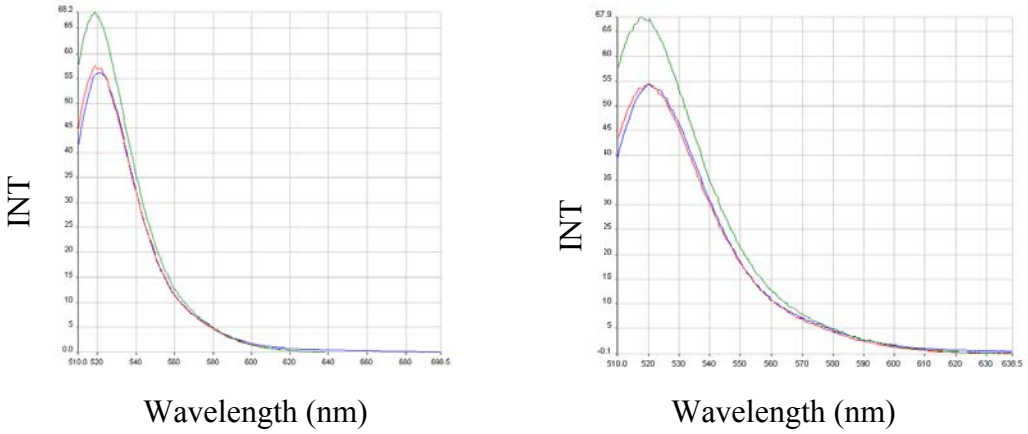


Figure 5-10. Original FRET spectra for the mixture GroEL(wt)$_{K242C}$ labeled with F5M and GroEL(wt)$_{N527C}$ labeled with TMR. *Left*: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. *Right*: blue: "protein only" group; red: group"protein + ADP"; green: "protein + ADP/ES" group.

5.6 There was some donor (and acceptor) emission peak shifting from FRET spectra of GroEL(wt)$_{K242C}$ and GroEL(cf)$_{K242C}$ mutants

For GroEL(wt)$_{K242C}$ labeled with F5M and TMR by Method D'A', FRET results showed that the donor and acceptor emission peaks shifted by addition of nucleotides and GroES (Figure 5-11).

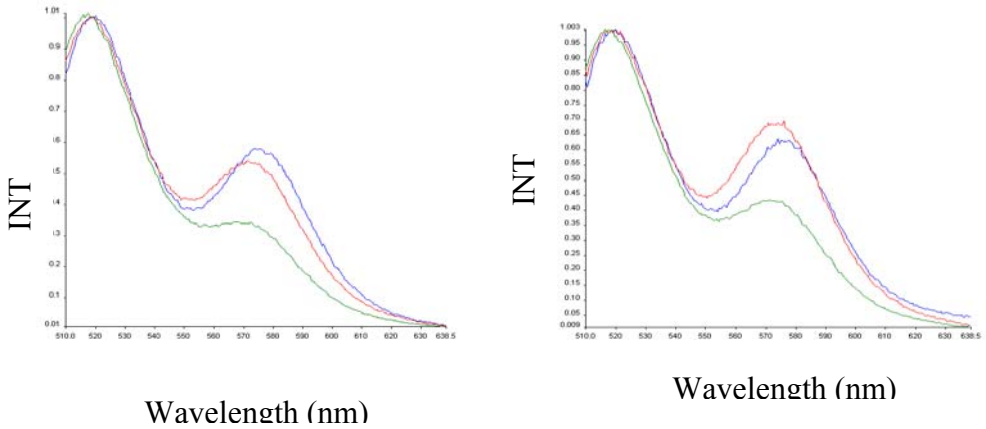


Figure 5-11. Normalized FRET spectra for GroEL(wt)$_{K242C}$ labeled with F5M and TMR by Method D'A'. *Left*: blue: "protein only" group; red: "protein + ATP" group; "protein + ATP/ES" group. *Right*: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

For GroEL(cf)$_{K242C}$, which was labeled with F5M and TMR by Method D'A', FRET spectra showed that the donor emission peaks also shifted (Figure 5-12).

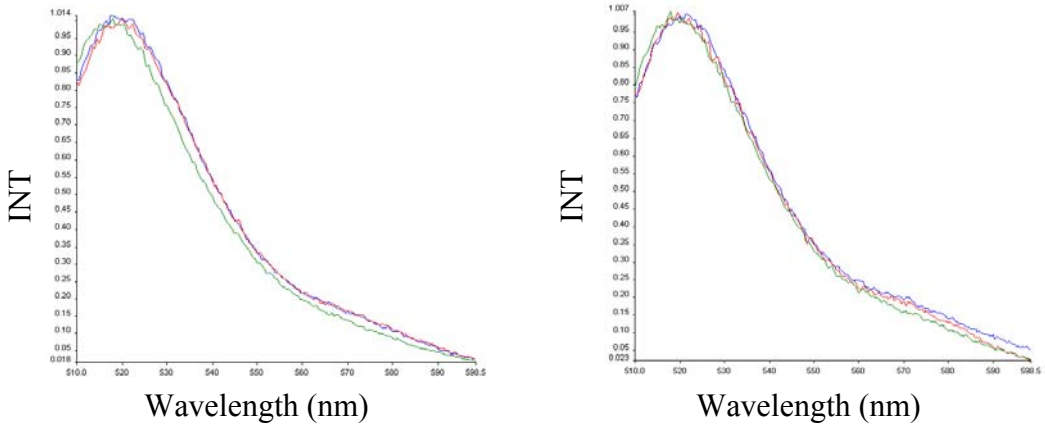


Figure 5-12. Normalized FRET spectra for GroEL(cf)$_{K242C}$ labeled with F5M and TMR by Method D'A'. *Left*: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. Right: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

For the same protein, labeled with F5M and TMR by co-mingling, the donor emission peaks shifted again with nucleotides and GroES addition (Figure 5-13).

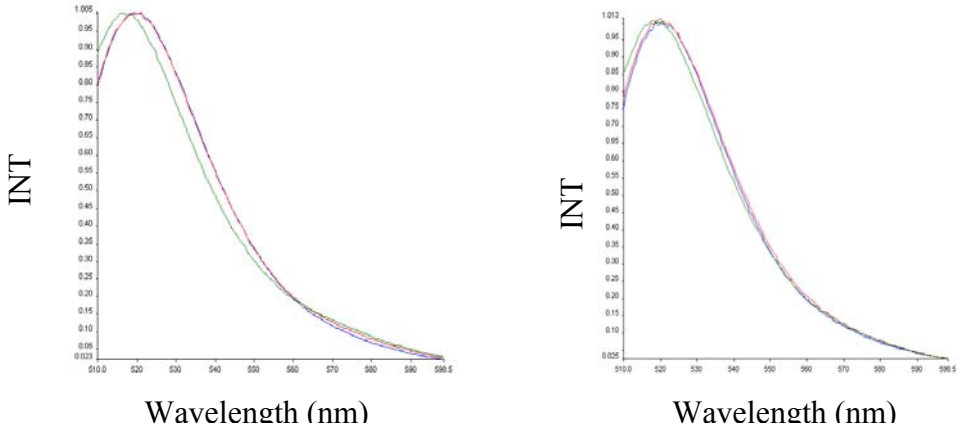


Figure 5-13. Normalized FRET spectra for GroEL(cf)$_{K242C}$ labeled with F5M and TMR by co-mingling. Left: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. Right: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

The most probable explanation for the spectral shifting is that the residue 242 is in helix H which is the hydrophobic SP binding site and may be interacting with this surface. So SP (denatured α-LA) was added to the labeled "protein only" group prior to addition of nucleotides and GroES. The spectra showed that addition of SP did not change E compared with that for labeled "protein only" group, and E increased by addition and ATP and ES (Figure 5-14).

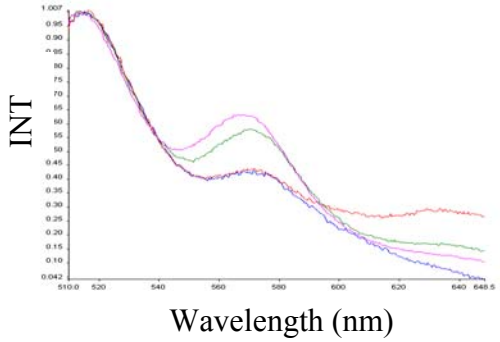


Figure 5-14. Normalized FRET spectra for GroEL(cf)$_{K242C}$ labeled with F5M and TMR upon SP addition. Blue: "protein only" group; red: "protein + SP" group; green: "protein + SP/ATP" group; pink: "protein + SP/ATP/ES" group.

These results did not confirm our expectation, however we hypothesize that it is possible the SP binding is not strong enough to displace a dye that is interacting with the SP binding site.

5.7 Positions of 242 and 138 might be a pair which could be labeled with donor and acceptor to monitor GroEL allosteric transition

I removed two native Cys sited (458 and 519) on each subunit so mutant $GroEL_{K242C/C458A/C519A}$ only has two Cys sites (242 and 138) left on each subunit. $GroEL_{K242C/C458A/C519A}$ was labeled with F5M and TMR and gained an average of 1 donor and 1 acceptor per ring, the spectra corresponded with our initial hypothesis: addition of ATP decreased the acceptor emission peak; addition of GroES had additional decrease (Figure 5-15).

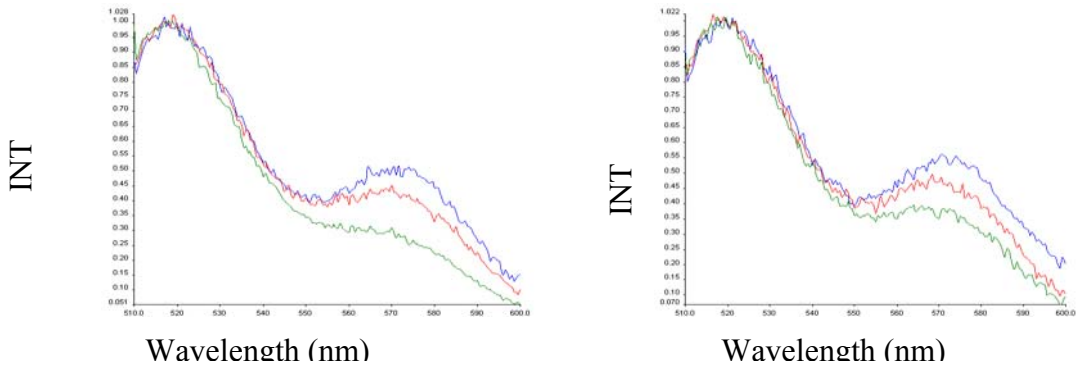


Figure 5-15. Normalized FRET spectra for GroEL(wt)$_{K242C/C458A/C519A}$ labeled with F5M and TMR. *Left*: blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group. *Right*: blue: "protein only" group; red: "protein + ADP" group; green: "protein + ADP/ES" group.

5.8 The fluorescent pair of F5M and TMR are not be a suitable pair for some GroEL mutants

From $GroEL(cf)_{E315C}$ labeled with F5M, a spectra change that resembled the energy transfer previously observed occurred on addition of ATP and GroES for the labeled protein (Figure 5-16). This should not happen since no acceptor was present.

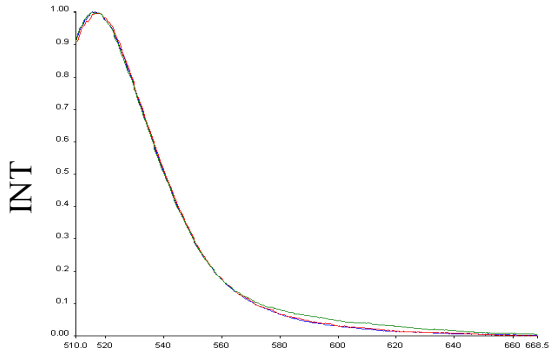


Figure 5-16. Normalized FRET spectra for GroEL(cf)$_{E315C}$ labeled with F5M by Method B'. Blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group.

For GroEL(cf)$_{E315C}$ labeled with acceptor (TMR), addition of ATP and GroES also increased the emission peaks (Figure 5-17), which was not expected.

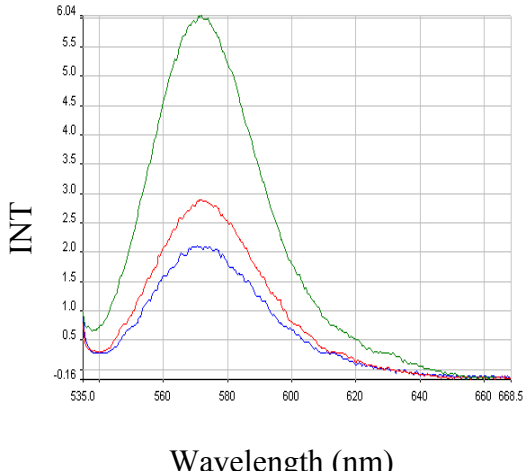


Figure 5-17. Original FRET spectra for GroEL(cf)$_{E315C}$ labeled with TMR by Method B'. Blue: l"protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group.

For simply mixing GroEL(cf)$_{E315C}$-F5M and GroEL(wt)$_{E315C}$-TMR, there was FRET occurring (Figure 5-18), that should not happen since the donors and the acceptors were on different GroEL 14 mers.

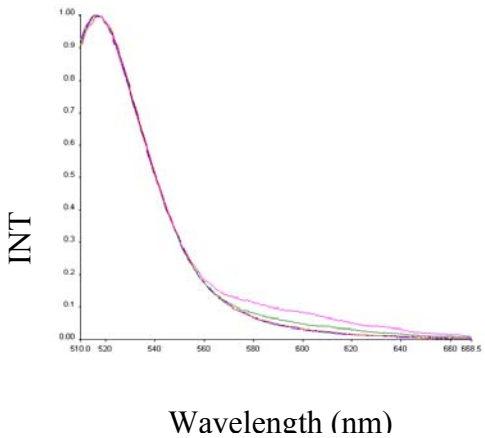


Figure 5-18. Normalized FRET spectra for a single mixture of GroEL(cf)$_{E315C}$ -F5M and GroEL(cf)$_{E315C}$ –TMR. Blue: GroEL$_{E315C}$-F5M only group; red: GroEL$_{E315C}$-F5M + ATP; green: GroEL$_{E315C}$-F5M + ATP/ES; pink: GroEL$_{E315C}$-F5M + ATP/ES + GroEL$_{E315C}$-TMR.

We anticipate that F5M and TMR are so sensitive to pH, hydrogen bonding and other environmental changes, that inclusion in the GroEL system is complicated, so F5M and TMR may not be the best dyes to monitor GroEL allosteric change.

5.9 Cy3 and Cy5, Alexa fluor 488 and Alexa fluor 546 are good labeling pairs for the GroEL system

When GroEL(wt)$_{K242C/N527C}$ was labeled with Cy3 and Cy5, even though each GroEL subunit has 5 Cys sites, with lower labeling extent (average 1.3 donors and 1.5 acceptors on each subunit), FRET spectra looked like that FRET efficiency (E) decreased in presence of ATP and GroES (Figure 5-9). These results met the expectation.

Since GroEL(cf)$_{E315C}$ only has one Cys site (residue 315 in apical domain), it prevents the intra-subunit and inter-ring effects from interfering with FRET signals. The FRET efficiency change appears to represent its allosteric change while minimizing photochemical and photo physical effects. When this mutant was labeled with Alexa fluor 488 and Alexa fluor 546, FRET spectra showed that E decreased on addition of ATP and GroES (Figure 5-19), which matched with the expectation.

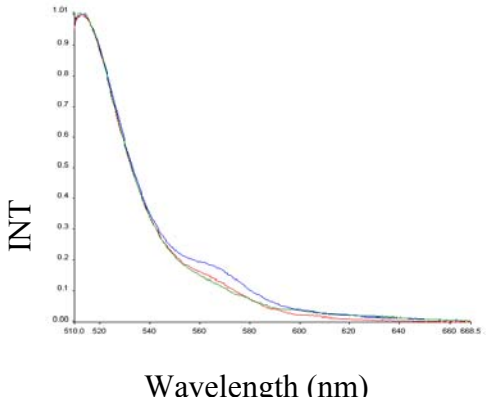


Figure 5-19. Normalized FRET spectra for GroEL(cf)$_{E315C}$ labeled with Alexa fluor 488 and Alexa fluor 546 by co-mingling. Blue: "protein only" group; red: "protein + ATP" group; green: "protein + ATP/ES" group.

It appears that Cy3 and Cy5, and even more so Alexa fluor 488 and Alexa fluor 546 were good labeling pairs for the GroEL system.

Chapter 6

Summary

In the presence of ATP and GroES, GroEL transitions from T to R and R' state. To follow GroEL allosteric change, several GroEL mutants with Cys sites were created by site-directed mutagenesis. When these GroEL mutants were labeled with dyes, FRET experiment could be performed.

The spectra showed that no FRET occurs in individual GroEL 14-mers, since the distance between 2 GroEL 14-mers is bigger than the distance in which FRET may occur. Also it is found that GroEL wild type may be labeled with dyes under some labeling conditions, such as changing the ratio of the dye concentration to the GroEL monomer concentration, and adjusting the incubation time, pH, etc. It is reported that $GroEL(wt)_{K242C/N527C}$ is not the best mutant for monitoring the GroEL structural change because of its wild type background. In GroEL, positions of 242 and 138 might be a pair which could be labeled F5M and TMR to monitor allosteric change under optimal labeling conditions, for example, each ring gets an average of one donor and one acceptor. This mutant ($GroEL(cf)_{K242C/C458A/C519C}$) represents a double-subunit strategy, and its FRET signals come from the intra-subunit effect. On the other hand, the mutant $GroEL(cf)_{E315C}$ labeled with Alexa fluor 488 and Alexa fluor 546 is a promising for following the GroEL structural change, and it represents a single-subunit strategy and inter-subunit effect.

For the future study, quenching experiments might be done to explain the problems from the spectra of mutant $GroEL(cf)_{K242C}$. By adding the quencher (eg: I^{-1}) to the labeled GroEL, the fluorescein data might give a complete picture for this system.

Appendix: The remaining GroEL mutants mutagenesis results

A.1 GroEL(wt)$_{K242C}$ mutant

The cloning of mutant GroEL(wt)$_{K242C}$ was first checked by restriction enzyme (*FspI*) digest (Figure A-1). The mutagenesis was designed so as to create an additional *Fsp I* site. This result matched with the primers' design which created one more *FspI* cleavage site. The mutagenesis result of GroEL(wt)$_{K242C}$ mutants was confirmed by DNA sequencing (University of Maryland DNA sequencing facility).

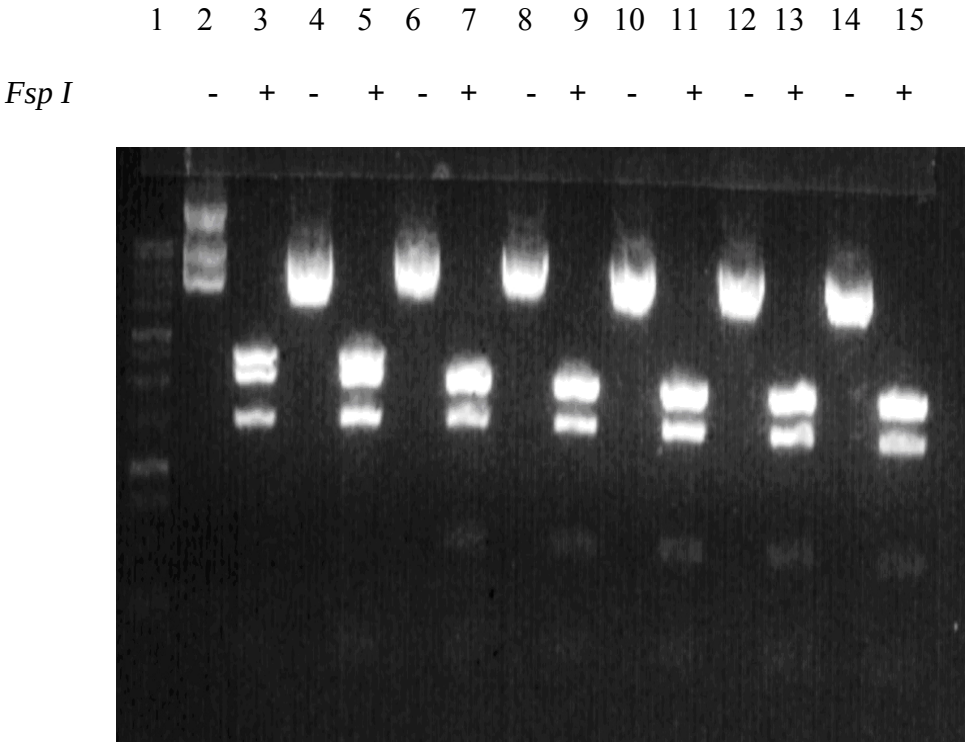


Figure A-1. Result for mutagenesis of GroEL(wt)$_{K242C}$ digested with *FspI*. (from *left* to *right*) Lane 1: 1 kb DNA marker. Lanes 2 and 3: GroEL(wt) samples. Lanes 4-15: GroEL(wt)$_{K242C}$ samples. Lanes 2, 4, 6, 8, 10, 12 and 14: not treated with *FspI*, and lanes 3, 5, 7, 9, 11, 13 and 15: treated with *FspI*.

A.2 GroEL(wt)$_{N527C}$ mutant

Restriction enzyme (*PvuII*) was used to check the mutagenesis result of GroEL(wt)$_{N527C}$ mutant (Figure A-2). Because of the primers' design, one *PvuII* cutting site was removed. From the agarose gel result, there were some differences between GroEL(wt) and GroEL$_{N527C}$ groups after treated with *PvuII*. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{N527C}$.

	1	2	3	4	5	6	7	8	9
Pvu II		-	+	-	+	-	+	-	+

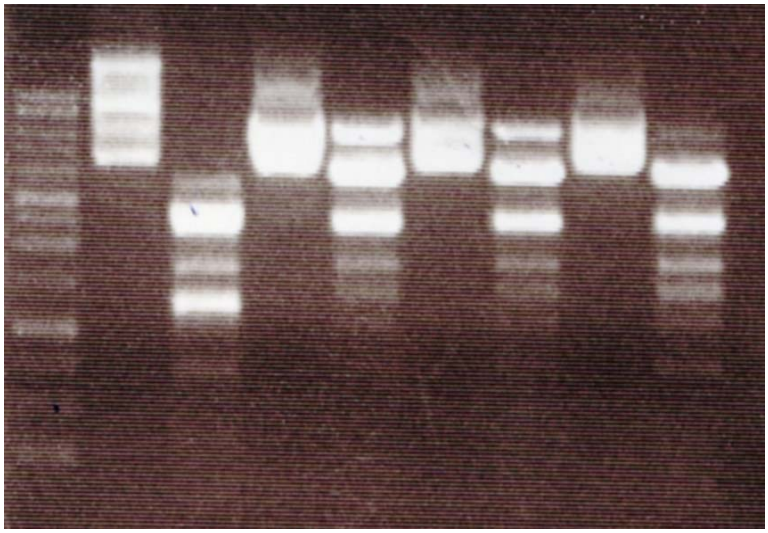


Figure A-2. Result for mutagenesis of GroEL(wt)$_{N527C}$ digested with *PvuII*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: GroEL(wt) samples. Lanes 4-9: GroEL$_{N527C}$ samples. Lanes 2, 4, 6 and 8: not treated with *PvuII*, and lanes 3, 5, 7 and 9: treated with *PvuII*.

A.3 GroEL(wt)$_{K242C/N527C}$ mutant

Mutagenesis result of GroEL(wt)$_{K242C/N527C}$ with GroEL(wt)$_{K242C}$ as background was checked with *PvuII* (Figure A-3). After digested by *PvuII*, there were some differences among the control group and GroEL(wt)$_{K242C/N527C}$ groups. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{K242C/N527C}$.

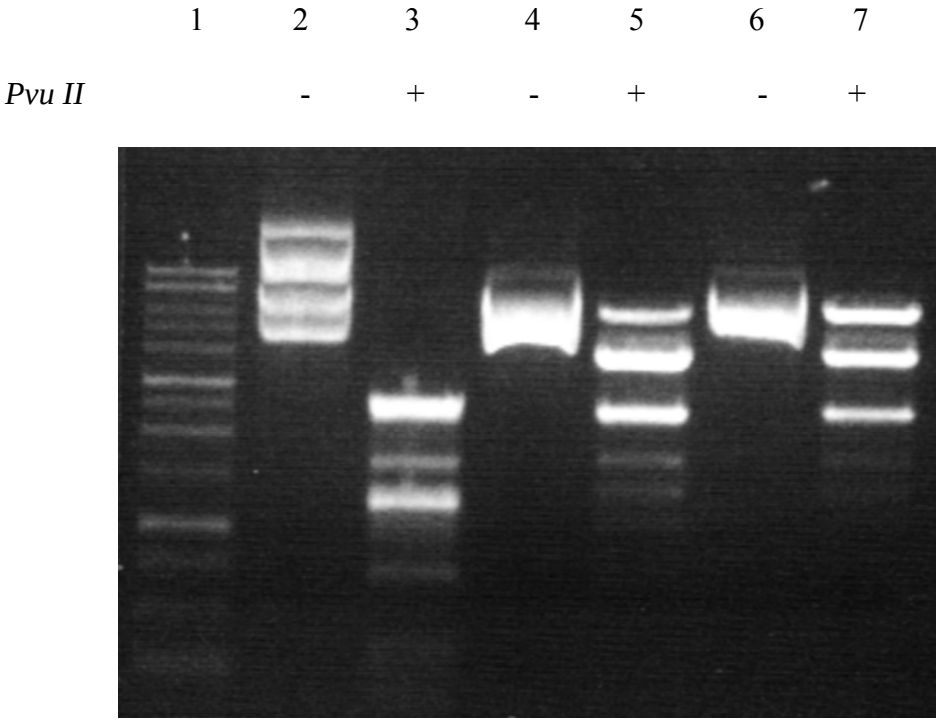


Figure A-3. Result for mutagenesis of GroEL(wt)$_{K242C/N527C}$ digested with *PvuII*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: GroEL(wt) samples. Lanes 4-7: GroEL(wt)$_{K242C/N527C}$ samples. Lanes 2, 4, and 6: not treated with *PvuII*, and lanes 3, 5 and 7: treated with *PvuII*.

A.4 GroEL(wt)$_{K242C/C458A}$ mutant

Mutagenesis result of GroEL(wt) $_{K242C/C458A}$ with GroEL(wt)$_{K242C}$ as background was checked with *Nae I* (Figure A-4). Theoretically, control (GroEL(wt)) should have one *Nae I* cutting site, and GroEL(wt)$_{K242C/C458A}$ has two *Nae I* cutting sites. The gel's result matched with the expectation. Also DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{K242C/C458A}$.

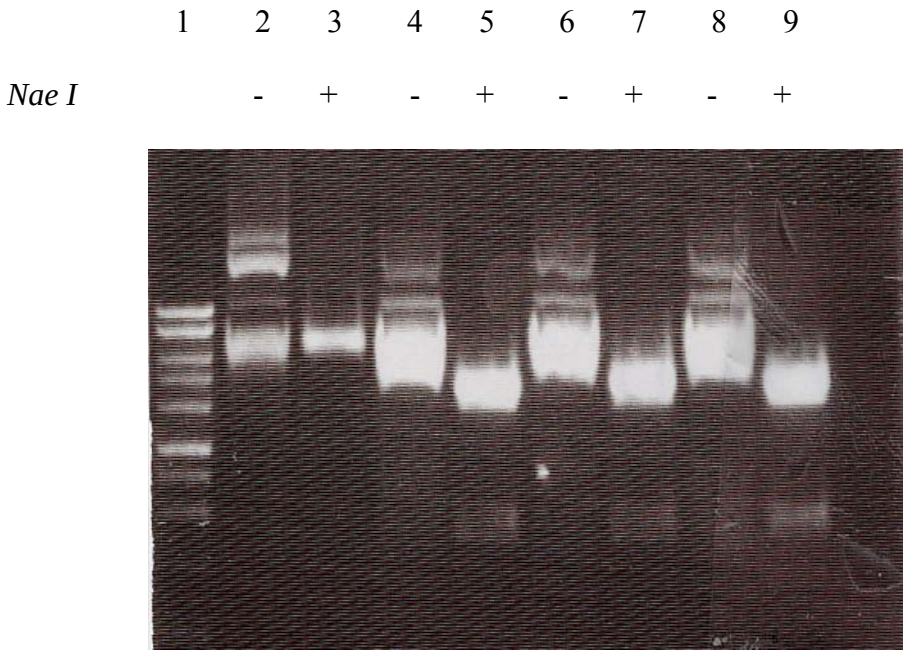


Figure A-4. Result for mutagenesis of GroEL(wt)$_{K242C/C458A}$ digested with *NaeI*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: GroEL(wt) samples. Lanes 4-9: GroEL(wt)$_{K242C/C458A}$ samples. Lanes 2, 4, 6 and 8: not treated with *Nae I*, and lanes 3, 5, 7 and 9: treated with *Nae I*.

A.5 GroEL(wt)$_{K242C/C458A/C519A}$ mutant

Mutagenesis result of GroEL(wt) $_{K242C/C458A/C519A}$ using GroEL(wt)$_{K242C/C458A}$ as background was checked with *Nsi I* (Figure A-5). As expected, the gel result showed that there was one *Nsi I* cutting site for control, no *Nsi I* cutting site for GroEL(wt)$_{K242C/C458A/C519A}$. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{K242C/C458A/C519A}$.

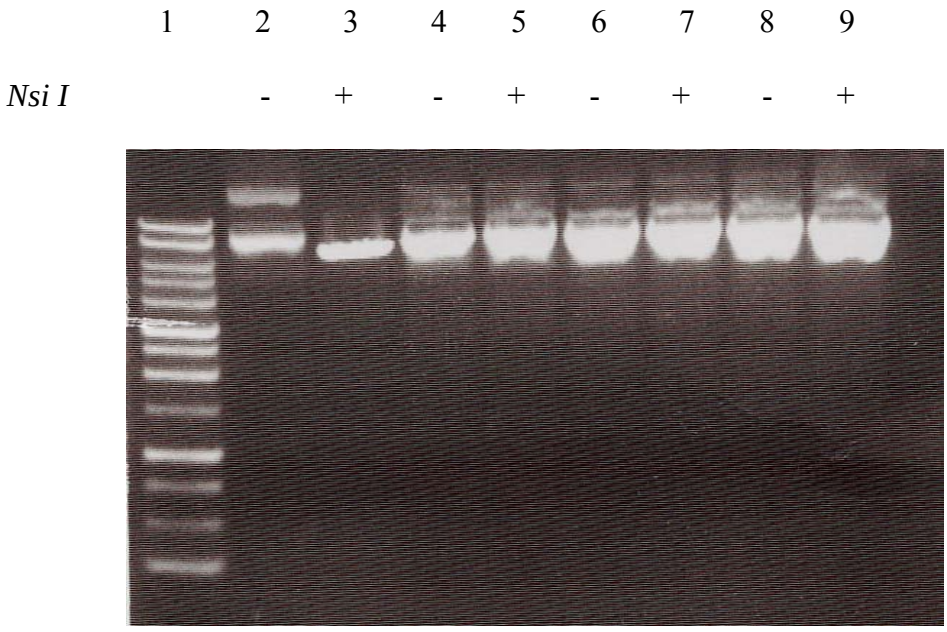


Figure A-5. Result for mutagenesis of GroEL(wt)$_{K242C/C458A/C519A}$ digested with *Nsi I*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: GroEL(wt)$_{WT}$ samples. Lanes 4-9: GroEL(wt)$_{K242C/C458A/C519A}$ samples. Lanes 2, 4, 6 and 8: not treated with *Nsi I*, and lanes 3, 5, 7 and 9: treated with *Nsi I*.

A.6 GroEL(wt)$_{C138S}$ mutant

Mutagenesis result of GroEL(wt)$_{C138S}$ from GroEL(wt) background was checked with *Bse YI* (Figure A-6, lanes 7 & 9). As expected, gel result showed that there were three *Bse YI* cutting sites for GroEL(wt)$_{C138S}$. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{C138S}$.

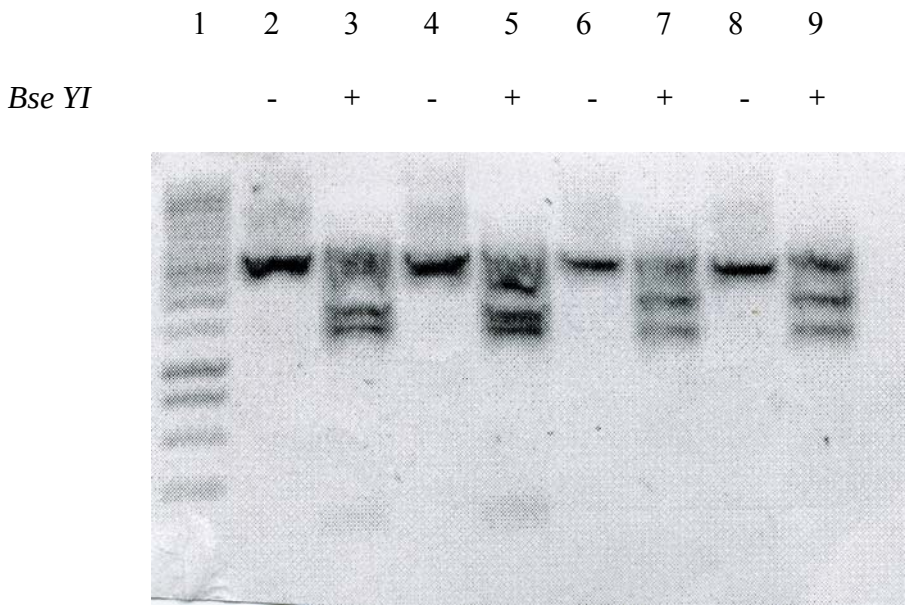


Figure A-6. Result for mutagenesis of GroEL(wt)$_{C138S}$ and GroEL(sr)(wt)$_{C138S}$ digested with *BseYI*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2-5: GroEL(sr)$_{C138S}$ samples. Lanes 6-9: GroEL(wt)$_{C138S}$ samples. Lanes 2, 4, 6 and 8: not treated with *Bse YI*, and lanes 3, 5, 7 and 9: treated with *Bse YI*.

A.7 GroEL(wt)$_{C138S/C519S}$ mutant

Mutagenesis result of GroEL(wt)$_{C138S/C519S}$ using GroEL(wt)$_{C138S}$ as background was checked with *Nco I* (Figure A-7). As expected, the gel result showed that there was one *Nco I* cutting site for control, two *Nco I* cutting sites for GroEL(wt)$_{C138S/C519S}$. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(wt)$_{C138S/C519}$.

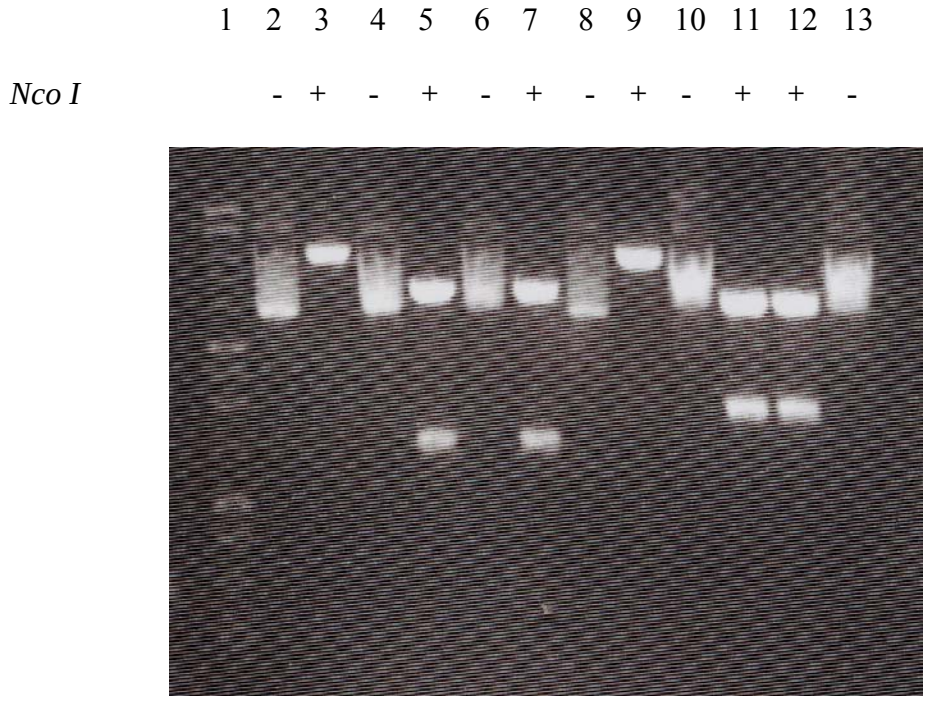


Figure A-7. Result for mutagenesis of GroEL(wt)$_{C138S/C519S}$ and GroEL(sr)$_{C138S/C519S}$ digested with *NcoI*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(wt)$_{C138S}$ samples). Lanes 4-7: GroEL(wt)$_{C138S/C519S}$ samples. Lanes 8 and 9: controls (GroEL(sr)$_{C138S}$ samples). Lanes 10-13: GroEL(sr)$_{C138S/C519S}$ samples. Lanes 2, 4, 6, 8, 10 and 13: not treated with *Nco I*, and lanes 3, 5, 7, 9, 11 and 12: treated with *Nco I*.

A.8 GroEL(cf) mutant

Mutagenesis result of GroEL(cf) using GroEL(wt)$_{C138S/C519S}$ as background was checked with *Eco RI* (Figure A-8). The gel result showed that there was one more *Eco RI* cutting site for GroEL(cf) than for the control, which matched with the design. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(cf).

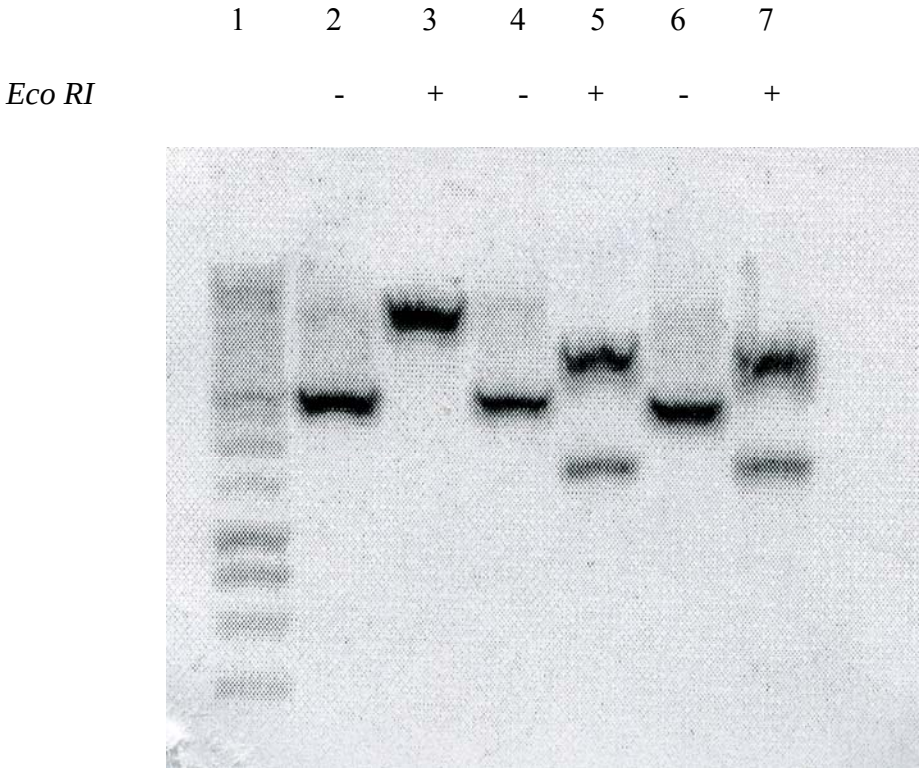


Figure A-8. Result for mutagenesis of GroEL(cf) digested with *Eco RI*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(wt)$_{C138S/C519S}$ samples). Lanes 4-7: GroEL(cf) samples. Lanes 2, 4, and 6: not treated with *Eco RI*, and lanes 3, 5 and 7: treated with *Eco RI*.

A.9 GroEL(cf)$_{E315C}$ mutant

Mutagenesis result of GroEL(cf)$_{E315C}$ using GroEL(cf) as background was checked with *Bln I* (Figure A-9). As expected, the gel result showed that there was one Bln *I* cutting site for GroEL(cf)$_{E315C}$ and no *Bln I* cutting site for the control. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(cf)$_{E315C}$.

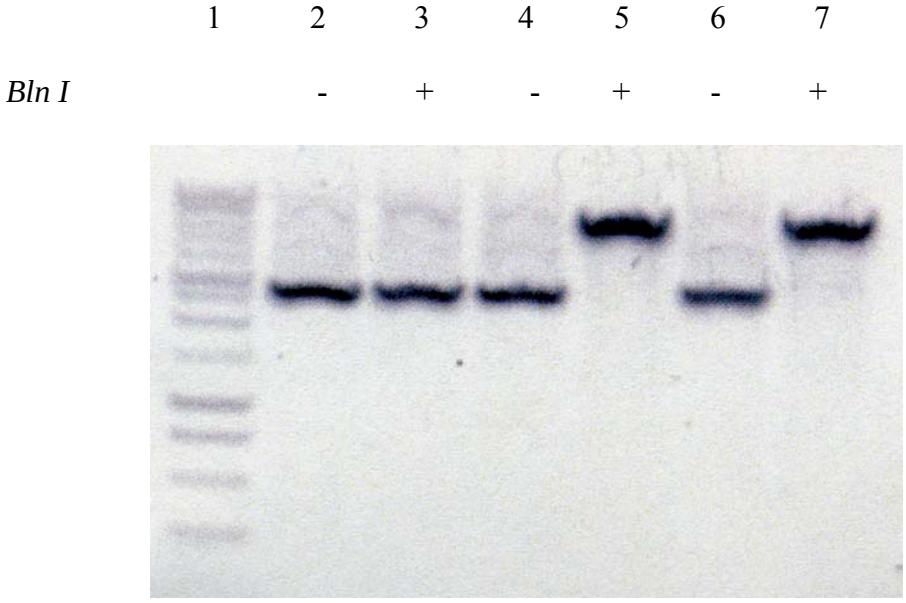


Figure A-9. Result for mutagenesis of GroEL(cf)$_{E315C}$ digested with *Bln I*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(cf) samples. Lanes 4, 5, 6 and 7: GroEL(cf)$_{E315C}$ samples. Lanes 2, 4 and 6: not treated with *Bln I*, lanes 3, 5 and 7: treated with *Bln I*.

A.10 GroEL(cf)$_{S217C}$ mutant

Mutagenesis result of GroEL(cf)$_{S217C}$ using GroEL(cf) as background was checked with *BsmI* (Figure A-10, lane 5). As expected, the gel result showed that there were two *Bsm I* cutting sites for GroEL(cf)$_{S217C}$ and one *Bsm I* cutting site for the control. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(cf)$_{S217C}$.

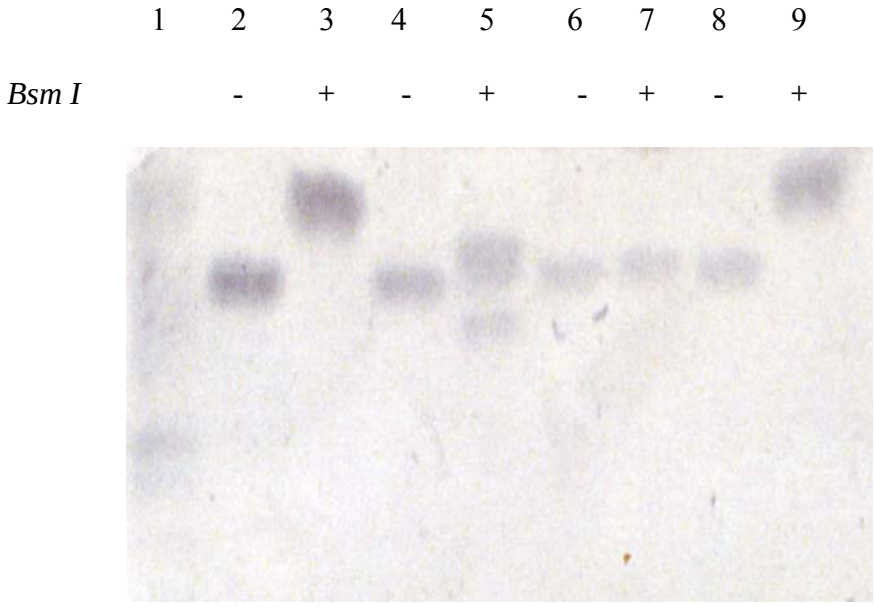


Figure A-10. Result for mutagenesis of GroEL(cf)$_{S217C}$ and GroEL(sr)$_{C138S/C519S/C458S/S217C}$ digested with *Bsm I*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(cf) samples). Lanes 4 & 5: GroEL(cf)$_{S217C}$ samples. Lanes 6 and 7: controls (GroEL(sr)$_{C138S/C519S/C458S}$ samples). Lanes 8 and 9: GroEL(sr)$_{C138S/C519S/C458S/S217C}$ samples . Lanes 2, 4, 6 and 8: not treated with *Bsm I*, lanes 3, 5, 7 and 9: treated with *Bsm I*.

A.11 GroEL(cf)$_{K321C}$ mutant

Mutagenesis result of GroEL(cf)$_{K321C}$ using GroEL(cf) as background was checked with *Stu I* (Figure A-11, lane 5). As expected, the gel result showed that there was one *Stu I* cutting site for GroEL(cf)$_{K321C}$ and no *Stu I* cutting site for the control. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(cf)$_{K321C}$.

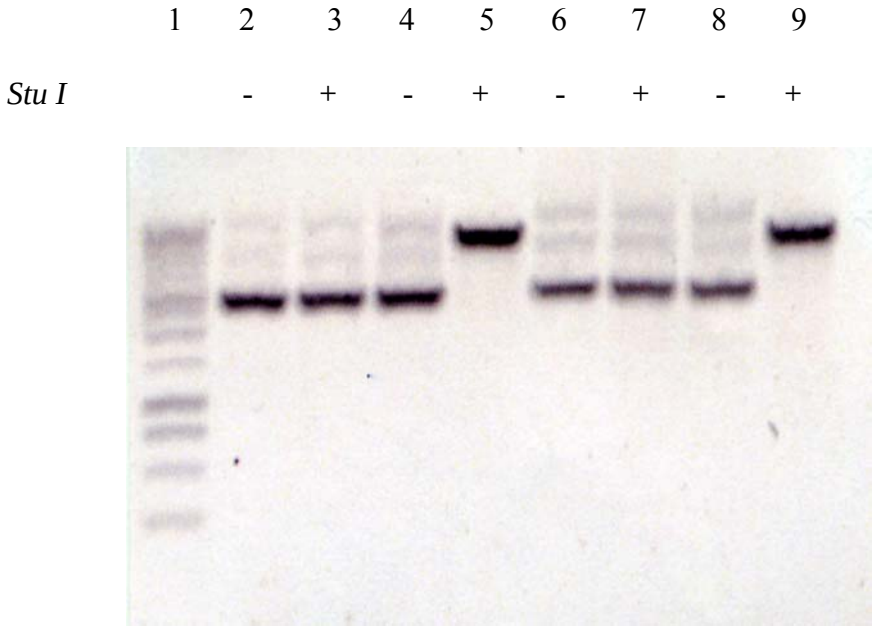


Figure A-11. Result for mutagenesis of GroEL(cf)$_{K321C}$ and GroEL(sr)$_{C138S/C519S/C458S/K321C}$ digested with Stu I. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(cf) samples). Lanes 4 & 5: GroEL(cf)$_{K321C}$ samples. Lanes 6 and 7: controls (GroEL(sr)$_{C138S/C519S/C458S}$ samples). Lanes 8 and 9: GroEL(sr)$_{C138S/C519S/C458S/K321C}$ samples . Lanes 2, 4, 6 and 8: not treated with *Stu I*, lanes 3, 5, 7 and 9: treated with *Stu I*.

A.12 GroEL(sr)$_{C138S}$ mutant

Mutagenesis result of GroEL(sr)$_{C138S}$ using GroEL(sr) as background was checked with *Bse YI* (Figure A-12, lane 3 & 5). DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(sr)$_{C138S}$.

A.13 GroEL(sr)$_{C138S/C519S}$ mutant

Mutagenesis result of GroEL(sr) $_{C138S/C519S}$ using GroEL(sr)$_{C138S}$ as background was checked with *Nco I* (FigureA-13, lanes 5 & 7). As expected, the gel result showed that there were one *Nco I* cutting site for control, two *Nco I* cutting sites for GroEL(sr)$_{C138S/C519S}$. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL$_{C138S/C519S}$.

A.14 GroEL(sr)$_{C138S/C519S/C458S}$ mutant

Mutagenesis result of GroEL(sr)$_{C138S/C519S/C458S}$ using GroEL(sr)$_{C138S/C519S}$ as background was checked with *Eco RI* (Figure A-13). The gel result showed that there was one more *Eco RI* cutting site for GroEL$_{C138S/C519S/C458S}$ than for the control, which matched with the design. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(sr)$_{C138S/C519S/C458S}$.

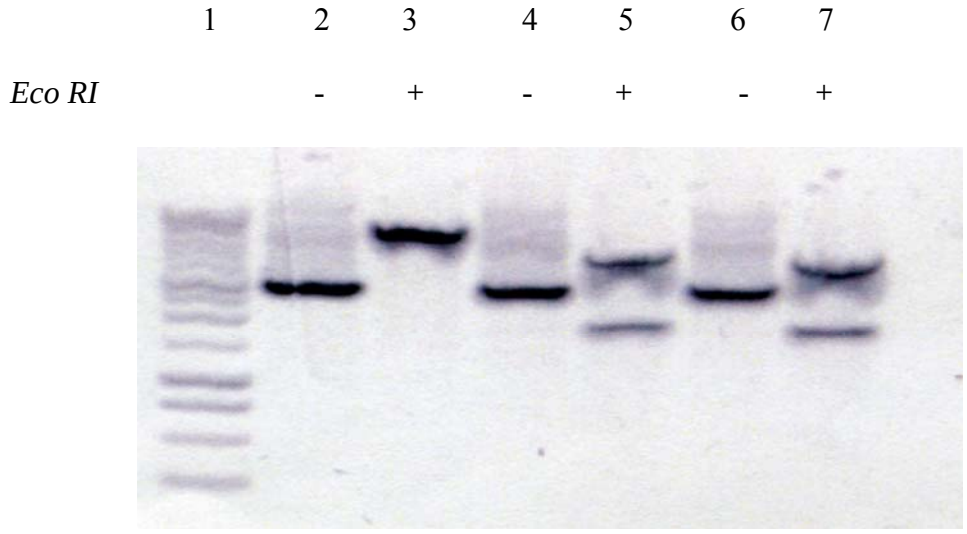


Figure A-12. Result for mutagenesis of GroEL(sr)$_{C138S/C519S/C458S}$ digested with *Eco RI*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(sr)$_{C138S/C519S}$ samples). Lanes 4-7: GroEL(sr)$_{C138S/C519S/C458S}$ samples. Lanes 2, 4,and 6: not treated with *Eco RI*, and lanes 3, 5 and 7: treated with *Eco RI*.

A.15 GroEL(sr)$_{C138S/C519S/C458S/E315C}$ mutant

Mutagenesis result of GroEL(sr) $_{C138S/C519S/C458S/E315C}$ from GroEL(sr)$_{C138S/C519S/C458S}$ background was checked with *Bln I* (Figure A-13). As expected, the gel result showed that there was one *Bln I* cutting site for GroEL$_{C138S/C519S/C458S/E315C}$ and no *Bln I* cutting site for the control. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(sr)$_{C138S/C519S/C458S/E315C}$.

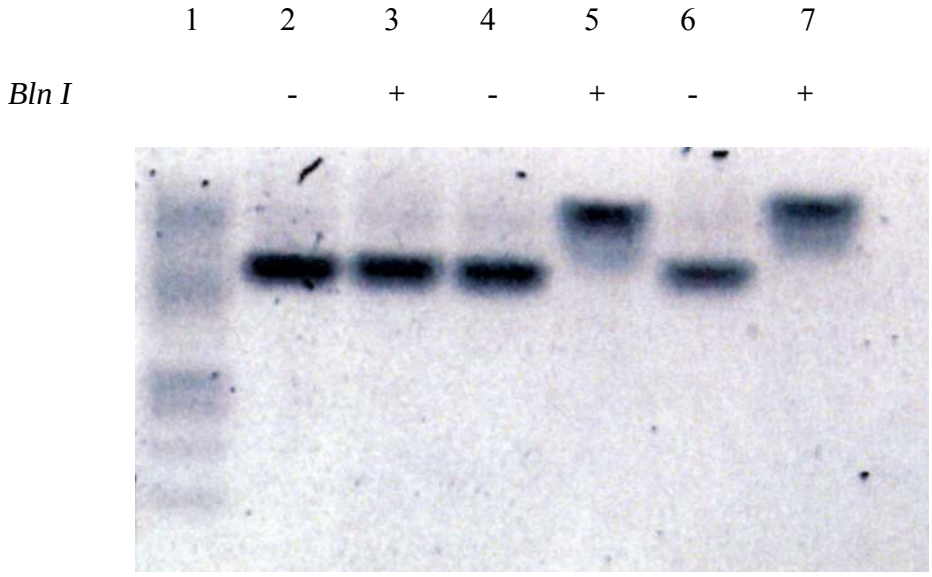


Figure A-13. Result for mutagenesis of GroEL(sr)$_{C138S/C519S/C458S/E315C}$ digested with *Bln I*. (From *left* to *right*) Lane 1: DNA marker. Lanes 2 and 3: controls (GroEL(sr)$_{C138S/C519S/C458S}$ samples). Lanes 4, 5, 6 and 7: GroEL(sr)$_{C138S/C519S/C458S/E315C}$ samples. Lanes 2, 4 and 6: not treated with *Bln I*, lanes 3, 5 and 7: treated with *Bln I*.

A.16 GroEL(sr)$_{C138S/C519S/C458S/S217C}$ mutant

Mutagenesis result of GroEL(sr)$_{C138S/C519S/C458S/S217C}$ using GroEL(sr)$_{C138S/C519S/C458S}$ as background was checked with *BsmI* (Figure A-10, lane 9). The gel result showed that there was one more *Bsm I* cutting site for GroEL(sr)$_{C138S/C519S/C458S/S217C}$ than for the control, matched with the primers' design. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(sr)$_{C138S/C519S/C458S/S217C}$.

A.17 GroEL(sr)$_{C138S/C519S/C458S/K321C}$ mutant

Mutagenesis result of GroEL(sr)$_{138S/C519S/C458S/K321C}$ using GroEL(sr)$_{C138S/C519S/C458S}$ as background was checked with *Stu I* (Figure A-11, lane 9). As expected, the gel result showed that there was one *Stu I* cutting site for GroEL(sr)$_{138S/C519S/C458S/K321C}$ and no *Stu I* cutting site for the control. DNA sequencing (University of Maryland DNA sequencing facility) confirmed the success of the mutagenesis of GroEL(sr)$_{C138S/C519S/C458S/K321C}$.

References

1. Anfinsen, C. B. (1973). "Principles that govern the folding of protein chains." Science 181(96): 223-30.

2. Beissinger, M., Rutkat, K.& Buchner, J. (1999). "Catalysis, commitment and encapsulation during GroE-mediated folding." J Mol Biol 289, 1075-92

3. Braig, K., Z. Otwinowski, et al. (1994). "The crystal structure of the bacterial chaperonin GroEL at 2.8 A." Nature 371(6498): 578-86.

4. Buchner, J., M. Schmidt, et al. (1991). "GroE facilitates refolding of citrate synthase by suppressing aggregation." Biochemistry 30(6): 1586-91.

5. Buckle, A. M., R. Zahn, et al. (1997). "A structural model for GroEL-polypeptide recognition." Proc Natl Acad Sci USA 94(8): 3571-5.

6. Burston, S. G., N. A. Ranson, et al. (1995). "The origins and consequences of asymmetry in the chaperonin reaction cycle." J Mol Biol 249(1): 138-52.

7. Chatellier, J., A. M. Buckle, et al. (1999). "GroEL recognises sequential and non-sequential linear structural motifs compatible with extended beta-strands and alpha-helices." J Mol Biol 292(1): 163-72.

8. Chen, L. and P. B. Sigler (1999). "The crystal structure of a GroEL/peptide complex: plasticity as a basis for substrate diversity." Cell 99(7): 757-68.

9. Cheng, M. Y., F. U. Hartl, et al. (1989). "Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria." Nature 337(6208): 620-5.

10. Clark, A. C. and C. Frieden (1999). "The chaperonin GroEL binds to late-folding non-native conformations present in native Escherichia coli and murine dihydrofolate reductases." J Mol Biol 285(4): 1777-88.

11. Cliff, M. J., N. M. Kad, et al. (1999). "A kinetic analysis of the nucleotide-induced allosteric transitions of GroEL." J Mol Biol 293(3): 667-84.

12. Corrales, F.J. & Fersht,A.R. (1996). "Kinetic signigicance of GroEL14(GroES7)2 complexes in molecular chaperone activity." Folding Design 1, 265-73

13. Eisenstein, E., Reddy, P., and Fisher, M. T. (1998) "Overexpression, purification, and properties of GroES from Escherichia coli." Methods in Enzymology 290, 119-135

14. Farr G. W., Furtak K., Rowland M. B., Ranson N. A., Saibi H. R., et al. (2000). "Multivalent binding of nonnative SPs by the chaperonin GroEL." Cell 100:491-500

15. Fenton, W. A., Y. Kashi, et al. (1994). "Residues in chaperonin GroEL required for polypeptide binding and release." Nature 371(6498): 614-9.

16. Fenton, W. A. and A. L. Horwich (2003). "Chaperonin-mediated protein folding: fate of substrate polypeptide." Q Rev Biophys 36(2): 229-56.

17. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) "Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfoleded state depends on two chaperonin proteins and Mg-ATP." Nature 342, 884-889

18. Goloubinoff, P., A. A. Gatenby, et al. (1989). "GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in Escherichia coli." Nature 337(6202): 44-7.

19. Grallert, H., K. Rutkat, et al. (2000). "Limits of protein folding inside GroE complexes." J Biol Chem 275(27): 20424-30.

20. Grallert, H. and J. Buchner (2001). "Review: a structural view of the GroE chaperone cycle." J Struct Biol 135(2): 95-103.

21. Grason, J.P. (2003) “Allostery in GroEL: Its role in the refolding of protein substrates.” In Department of Chemistry and Biochemistry, University of Maryland College Park, College Park, MD

22. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) “Homologous plant and bacterial proteins chaperone oligomeric protein assembly. “ Nature 333, 330-334

23. Horwich, A. L., K. B. Low, et al. (1993). "Folding in vivo of bacterial cytoplasmic proteins: role of GroEL." Cell 74(5): 909-17.

24. Hunt, J. F., A. J. Weaver, et al. (1996). "The crystal structure of the GroES co-chaperonin at 2.8 A resolution." Nature 379(6560): 37-45.

25. Inbar, E. and A. Horovitz (1997). "GroES promotes the T to R transition of the GroEL ring distal to GroES in the GroEL-GroES complex." Biochemistry 36(40): 12276-81.

26. Itzhaki, L. S., D. E. Otzen, et al. (1995). "Nature and consequences of GroEL-protein interactions." Biochemistry 34(44): 14581-7.

27. Jackson, G. S., R. A. Staniforth, et al. (1993). "Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: implications for the mechanism of assisted protein folding." Biochemistry 32(10): 2554-63.

28. Kad, N. M., N. A. Ranson, et al. (1998). "Asymmetry, commitment and inhibition in the GroE ATPase cycle impose alternating functions on the two GroEL rings." J Mol Biol 278(1): 267-78.

29. Katsumata, K., A. Okazaki, et al. (1996). "Effect of GroEL on the re-folding kinetics of alpha-lactalbumin." J Mol Biol 258(5): 827-38.

30. Lakowicz, J. (1999) Principle of fluorescence spectroscopy.

31. Lin, Z., F. P. Schwartz, et al. (1995). "The hydrophobic nature of GroEL- substrate binding." J Biol Chem 270(3): 1011-4.

32. Lin, Z and Rye,H.S. (2006) "GroEL-Mediated Protein Folding: Making the Impossible, Possible. " Biochem Mol Biol 41(4),211-39

33. Mande, S. C., V. Mehra, et al. (1996). "Structure of the heat shock protein chaperonin-10 of Mycobacterium leprae." Science 271(5246): 203-7.

34. Mayhew, M., A. C. da Silva, et al. (1996). "Protein folding in the central cavity of the GroEL-GroES chaperonin complex." Nature 379(6564): 420-6.

35. Mendoza, J. A., T. Warren, et al. (1996). "The ATPase activity of chaperonin GroEL is highly stimulated at elevated temperatures." Biochem Biophys Res Commun 229(1): 271-4.

36. Motojima,F., et al. (2004) "Substrate polypeptide presents a load on the apical domains of the chaperonin GroEL." Proc Natl Acad Sci 101(42) 15005-12

37. Perrett, S., R. Zahn, et al. (1997). "Importance of electrostatic interactions in the rapid binding of polypeptides to GroEL." J Mol Biol 269(5): 892-901.

38. Preuss, M., J. P. Hutchinson, et al. (1999). "Secondary structure forming propensity coupled with amphiphilicity is an optimal motif in a peptide or protein for association with chaperonin 60 (GroEL)." Biochemistry 38(32): 10272-86.

39. Ranson, N. A., Farr, G. W., Roseman, A. M., Gowen, B., Fenton, W. A., Horwich, A. L., and Saibil, H. R. (2001) "ATP-bound states of GroEL captured by cryo-electron microscopy." Cell 107, 3869-879.

40. Richarme, G. and M. Kohiyama (1994). "Amino acid specificity of the Escherichia coli chaperone GroEL (heat shock protein 60)." J Biol Chem 269(10): 7095-8.

41. Robert, M.W.,et al. (1979) "Solvent perturbation of the fluorescein bound to specific antibody." J Biol Chem 254(5), 1684-1690

42. Ryba, N. J., M. A. Hoon, et al. (1993). "Rhodopsin mobility, structure, and lipid-protein interaction in squid photoreceptor membranes." Biochemistry 32(13): 3298-305.

43. Rye, H. S., A. M. Roseman, et al. (1999). "GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings." Cell 97(3): 325-38.

44. Saibil, H. (2000). "Molecular chaperones: containers and surfaces for folding, stabilising or unfolding proteins." Curr Opin Struct Biol 10(2): 251-8.

45. Sakikawa, C., H. Taguchi, et al. (1999). "On the maximum size of proteins to stay and fold in the cavity of GroEL underneath GroES." J Biol Chem 274(30): 21251-6.

46. Schmidt M, Buchner J, Todd M. J, Lorimer G. H. and Viitanen P. V. (1994)." On the role of groES in the chaperonin-assisted folding reaction. Three case studies." J Biol Chem. 8;269(14):10304-11

47. Sigler, P. B., Z. Xu, et al. (1998). "Structure and function in GroEL-mediated protein folding." Annu Rev Biochem 67: 581-608.

48. Thiyagarajan, P., S. J. Henderson, et al. (1996). "Solution structures of GroEL and its complex with rhodanese from small-angle neutron scattering." Structure 4(1): 79-88.

49. Thirumalai, D. and G. H. Lorimer (2001). "Chaperonin-mediated protein folding." Annu Rev Biophys Biomol Struct 30: 245-69.

50. Todd, M. J., P. V. Viitanen, et al. (1994). "Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding." Science 265(5172): 659-66.

51. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1993) "Hydrolysis of adenosine 5'-triphosphate by Escherichia coli GroEL: effects of GroES and potassium ion." Biochemistry 32, 8560-8567

52. Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., and Lorimer, G. H. (1990) "Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K+ dependent." Biochemistry 29, 5665-5671

53. Wang, Z., H. Feng, et al. (1999). "Basis of substrate binding by the chaperonin GroEL." Biochemistry 38(39): 12537-46.

54. Weissman, J. S., C. M. Hohl, et al. (1995). "Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES." Cell 83(4): 577-87.

55. Weissman, J. S., H. S. Rye, et al. (1996). "Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction." Cell 84(3): 481-90.

56. Werih, S.C.. (2006) Procedure for monomer mixing experiment.In Department of Chemistry and Biochemistry, University of Maryland College Park, College Park, MD

57. Xu, Z., A. L. Horwich, et al. (1997). "The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex." Nature 388(6644): 741-50.

58. Xu, Z. and P. B. Sigler (1998). "GroEL/GroES: structure and function of a two-stroke folding machine." J Struct Biol 124(2-3): 129-41.

59. Yifrach, O. and A. Horovitz (1995). "Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL." Biochemistry 34(16): 5303-8.