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Biophysical Characterization and the effect of Biotinylation on the Physical Stability of GroEL

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ABSTRACT

The molecular chaperone GroEL assists in re-folding misfolded polypeptides. Many human diseases are due to protein misfolding. Alzheimer's disease, Parkinson's disease, Huntington's disease, and certain types of cancers are examples of protein misfolding diseases. Molecular chaperones such as GroEL could play a role in treating these diseases. Recent studies have used biotinylated GroEL as a tool in various protein re-folding assays and the question has been posed if biotinylation destabilizes GroEL. The primary objective of this study was to directly compare the effect of biotinylation on the physical stability of GroEL. Far-UV circular dichroism, extrinsic fluorescence spectroscopy, and UV-Vis optical density spectroscopy were used to assess the physical stability of GroEL as a function of pH (3-8) and temperature (10°-87.5°C). The data collected from the aforementioned techniques were used to construct empirical phase diagrams (EPDs), which provide a global assessment of protein stability. Both wild-type and biotinylated GroEL were significantly destabilized at low pH (3-4) as both visible aggregates and significantly altered tertiary structure was observed. Four distinct phases were observed in both EPD's. The native state of both species of GroEL was similar (pH 5-8 and ≤ 60˚C) and the greatest stability was observed at pH 6 and pH 7. This study suggests biotinylation has an insignificant effect on the physical stability of GroEL.

Keywords: *GroEL, Biotinylation, Protein Stability, Biophysical Characterization*

INTRODUCTION

Proteins are unique biological molecules with various functions in living organisms. There are four levels of protein structure. They are composed typically of 20 different amino acids. The order of those amino acids is the primary structure of a polypeptide. Secondary and tertiary structures refer to the "folding" of one polypeptide. Quaternary structure refers to the relationship between multiple polypeptides to form a functional unit. In order for the structures to function properly the protein must fold correctly. Polypeptide chains tend to aggregate with each other due to exposed hydrophobic regions rather than form functional structures (Ellis and Minton, 2006). Aggregation of unfolding could be caused by an innate instability or environmental stress such as extreme temperature or pH change (Ellis and Minton, 2006). Many protein folding defects are from missense mutations which shift the equilibrium distribution from the native state to a partially denatured or abnormally activated species (Naik, et al., 2010). It is estimated that that 30%-50% of human diseases are due to protein folding defects (Naik, et al., 2010). Some of these diseases are Alzheimer's disease, Parkinson's disease, Huntington's, ALS, Cystic Fibrosis, various Cancers, and Diabetes.

A strategy used to combat protein misfolding is identifying the pharmacological chaperone to stabilize the native folded distribution (Naik, et al., 2010). The primary function of chaperone proteins is to assist in refolding of misfolded polypeptides *in vivo.* GroEL, an *Escherichia coli* molecular chaperone, is often studied due to its analogous properties to hsp 60, a mitochondrial chaperone. Studies have found 84 proteins which are dependent on GroEL, at least 13 of which are essential proteins (Ellis and Hartl, 1999). GroEL assists in the folding process by binding to GroES to form the GroE complex and encapsulating individual polypeptide chains one at a time inside a molecular cage (Ellis and Hartl 1999). The protein is allowed to continue to fold without the presence of outside factors while in this cage (Ellisand Hartl, 1999).

Recent studies have used the biotinylation of GroEL as an analytical tool in various protein re-folding assay. The question has been posed on the effects of biotinylation to the stability of GroEL under *in vitro* conditions. Biotinylated molecules are easily detected with streptavidin (SA) derivatives (e.g. fluorophore or horseradish peroxidase) or efficiently captured on Avidin/SA coated solid supports (magnetic beads, resins, chips, etc) (Elia, 2008). With the biotin label attached to GroEL the stability of the native state could be disturbed.

To test the stability of GroEL, with and without biotin, various characterization studies were conducted. Far-UV circular dichroism is used to detect changes in the secondary structure, due to the high optical activity of helical structures (Maddux, et al., 2011). Extrinsic fluorescence using 8-anilino-1-naphthalenesulfonate (ANS) is utilized to monitor tertiary structural changes. As the tertiary structure becomes altered hydrophobic areas are exposed which bind to the ANS dye. A spectrofluorometer

monitors the amount of ANS bound during a temperature ramp. UV-Vis absorption spectroscopy is used to test turbidity. The absorption is measured at 360 nm because proteins do not absorb much light at this wavelength, assuming a homogenous solution, so any absorption is due to optical density. Optical density is a term coined instead of absorbance which takes both absorbance and turbidity into consideration (Middaugh, 2005).

These characterization studies are hard to visualize separately so they are combined into an Empirical Phase Diagram (EPD). An EPD is a colored representation of the overall structural integrity and conformational stability of macromolecules in response to various environmental stresses (Kim, et al, 2012). After an EPD has been constructed for the two proteins the overall stability will be compared. The comparison will show whether the biotinylation affects the stability of GroEL. In this study, the effect of biotinylation on the physical stability of GroEL was analyzed.

MATERIALS AND METHODS

Citric Phosphate (CP) Buffer Preparation

The physical stability of GroEL was tested at seven pHs (3, 4, 5, 6, 7, 7.5, and 8). Seven 500 mL citric phosphate buffer solutions were made and brought to specified pH by adding 6 M HCl or 6 M NaOH drop-wise. They were made at a 0.15 ionic strength using NaCl. When correct pH had been reached the solution was sterile filtered with a syringe through a 0.45 µm filter. All physical stability measurements were obtained in 20 mM CP Buffer.

GroEL Extraction from Glycerol Solution

GroEL obtained from the Mark Fisher Laboratory at Kansas University Medical Center was stored in glycerol to keep the GroEL stable. To extract GroEL add equal volume of sterile refolding buffer (50mM TRIS, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EDTA) and protein. Then the solution was centrifuge at 4000xg in a 100 kDa centrifugal filter. The glycerol excess is removed and the same amount of refolding buffer previously added is added again. The glycerol excess is extracted once again removed. This procedure is repeated three more times.

UV-Vis Spectrophotometer for Concentration Determination of GroEL and Bio-GroEL

The concentration of GroEL was tested with a UV-Vis spectrophotometer. Using 20 mM CP buffer as a blank. A 0.05 microliter sample of GroEL and biotinylated GroEL were diluted into 750 µL of CP buffer. The reading observed at 350-400 nm with 1 nm steps using a single wavelength at 280 nm to determine concentration. Light scattering was corrected for by using scatter correction function. The concentration was found using Beer-Lamberts law.$Abs=(c)(\varepsilon)(l)$

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

Four samples were made: GroEL, Biotinylated GroEL, GroEL and Dithiothreitol (DTT), and Biotinylated GroEL and DTT. The pure samples had 0.5 mg/mL protein mixed with ultrapure water and 5 µL of 4x Lithium Dodecyl Sulfate (LDS) sample buffer. The total volume used was 20 µL. The samples with DTT were made in the same manner, but with one µL 50 mM DTT instead of one µL ultrapure water. The total volume used was 20 µL. The samples were the heated at 100°C for five minutes. The samples along with a molecular weight marker were then loaded into a NuPage 10% Bis-Tris Gel with a MOPS running buffer. The gel was run for 75 minutes at 150 V. After the gel was run it is washed three times with ultrapure water. The gel was stained for one hour with Coomassie brilliant blue. The samples were allowed to destain while slowly being shaken in ultrapure water overnight. A picture was then taken.

Far UV Spectroscopy for Analysis of Secondary Structure of GroEL and Bio-GroEL

GroEL and Biotinylated GroEL solutions were diluted to 0.1 mg/mL in 250 µL CP Buffer solution and mixed for 30 s using a vortex mixer. Samples were placed in 0.1 cm quartz cuvettes. All GroEL and Biotinylated GroEL samples were tested at each pH (3,5,6,7,7.5, and 8). Secondary structure was tested using a Chirascan plus Circular Dichroism. The wavelength 200-260 nm was observed with the bandwidth set at 1.0 with 1.0 nm steps and 0.500-s readings per point. To observe spectra no temperature ramp was performed. To test the melting curve the temperature was increased from 10°C to 87.5°C with 2.5°C increments and one-minute readings at each temperature. Wavelength 222 nm was analyzed to observe secondary structure stability. Buffer baselines were subtracted from all measurements.

UV Vis Spectophotometry for Analysis of Aggregation of GroEL and Bio-GroEL

GroEL and Biotinylated GroEL solutions were diluted to 0.1 mg/mL in 300 µL CP Buffer solution and centrifuged for 30 s using a vortex mixer. Samples were placed in 0.1 cm quartz cuvettes. All GroEL and Biotinylated GroEL samples were tested at each pH. A Varian Cary 100 Bio UV-Vis Spectrophotometer was used for all readings. The wavelength was set to 360 nm with 2.0 seconds taken for each reading. The temperature was set to start at 10°C and increase in 1.25°C increments to 87.5°C, and return to 25°C for final readings. The temperature was set to increase at a rate of 0.5°C/min holding for 1.00 min before taking measurement. Buffer baselines were subtracted

from all measurements.

Extrinsic Fluorescence Spectroscopy for Analysis of Tertiary Structure of GroEL and Bio-GroEL

GroEL and Biotinylated GroEL solutions were diluted to 0.05 mg/mL in 750 µL CP Buffer with 50 µM 1-anilino-8-napthalene sulfonate (ANS) and mixed by swirling. Solutions were placed in 10 mm quartz cuvettes. A PTI spectrofluorometer was used to test tertiary structure. Samples were excited at 372 nm and emission was recorded from 400-600 nm. All slits were set to 2 nm. Readings were taken every 1 nm. Temperature was increased from 10°C to 87.5°C in 2.5°C intervals. The temperature was set to increase at a rate of 0.5°C/min.

Data Analysis

The UV-Vis spectrophotometry data was analyzed in Microsoft Excel® and transferred into Origin 7® to visualize. The averages and standard deviations for all samples were found and plotted with error bars.

Far-UV Spectroscopy was analyzed by following the 222 nm wavelength. The results from the 222 nm wavelength were placed into Origin 7® to visualize.

Extrinsic Fluorescence Spectroscopy was analyzed by finding the mean spectral mass of each spectrum. The mean spectral mass was then placed into Origin 7® and visualized. The data was also normalized in Microsoft Excel® using min-max normalization.

Empirical Phase Diagrams were made using two separate methods: Three Color Index and Singular Value Decomposition. The physical stability averages were placed in the MiddaughSuite program which computed the Empirical Phase Diagrams.

RESULTS

A variety of methods, including UV-Vis absorption spectroscopy, far-UV circular dichroism spectroscopy (CD), and extrinsic fluorescence were used to assess the stability of the secondary structure, tertiary structure, and aggregation states of GroEL and Bio-GroEL over a pH and temperature gradient. Before these tests were run, the molecular weight of GroEL and Bio-GroEL were determined using a SDS-PAGE to ensure the molecular weight had not significantly changed. The SDS-PAGE is shown in Figure 1. The SDS-PAGE confirmed that both GroEL and Bio-GroEL had 57 kDa monomers.

The CD spectrum at 10°C is shown in Figure 2 and Figure 3. The CD signal suggests that both GroEL and Bio-GroEL are alpha-helical structures based on the minimums at approximately 208 nm and 222 nm. A pH dependence is shown in both proteins as the molar ellipticity increases as the pH increases.

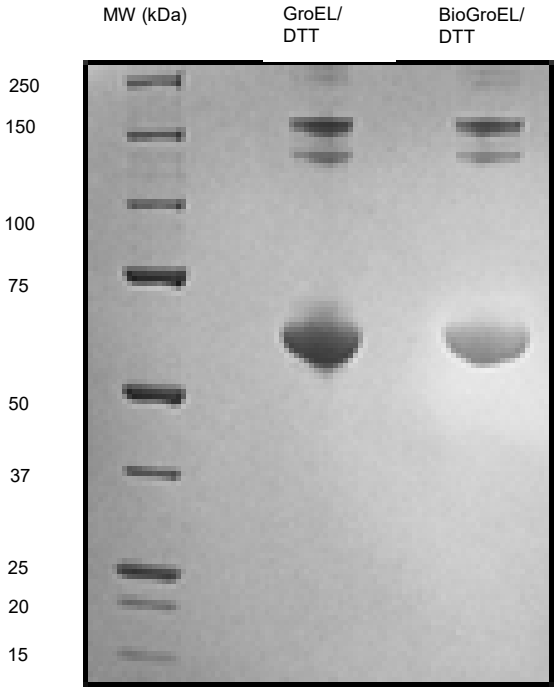

Figure 1. SDS-PAGE of GroEL and BioGroEL

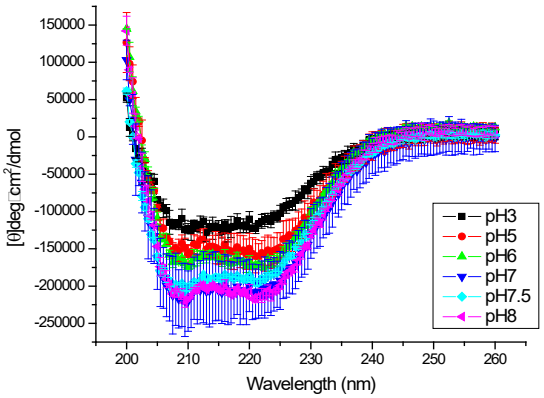

Figure 2. CD Spectra of GroEL at 10°C.

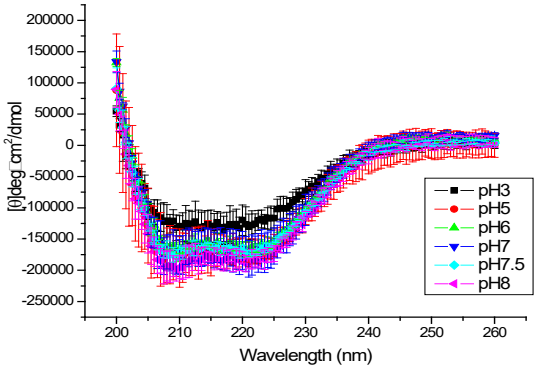

Figure 3. CD Spectra of Bio-GroEL at 10°C.

GroEL and Bio-GroEL were tested with a thermal melt. The wavelength 222 nm was monitored while increasing the temperature from 10°C to 87.5°C. Secondary structure of GroEL and Bio-GroEL is

structurally altered at pH 3. There is a noticeable transition from a stable state to a structurally altered state from approximately 60°C to 70°C. The secondary structure is compromised from 70°C to 87.5°C in both GroEL and Bio-GroEL. The secondary structure of GroEL and Bio-GroEL appear to be stable from 10°C to 60°C, and from pH5 to pH8. The biotinylation of GroEL does not appear to affect the stability of the secondary structure of the protein.

Figure 4. CD Thermal Melt of GroEL from 10°C to 87.5°C.

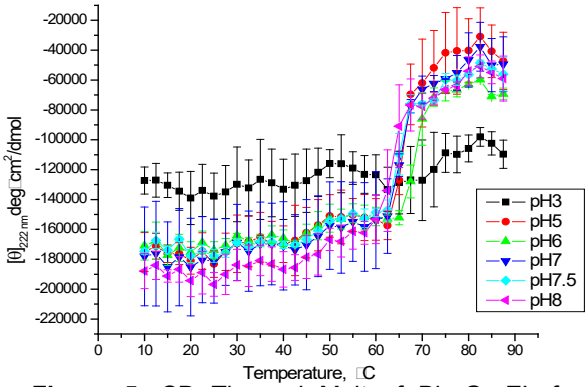

Figure 5. CD Thermal Melt of Bio-GroEL from 10°C to 87.5°C.

Extrinsic fluorescence measurements of GroEL and Bio-GroEL, with ANS as an extrinsic dye, were measured as a function of pH and temperature as well. The peak position of the ANS fluorescence emissions was found using a polynomial based method. Figure 6 and Figure 7 show the extrinsic fluorescence spectra at 10°C. At pH 3 the emission is much higher than at any other pH, this suggests the tertiary structure of GroEL and Bio-GroEL at pH 3 is structurally altered. Notice both the secondary structure and tertiary structure appear to be structurally altered at pH 3.

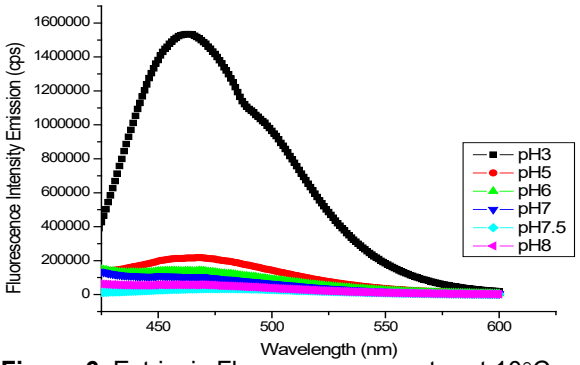

Figure 6. Extrinsic Fluorescence spectra at 10°C.

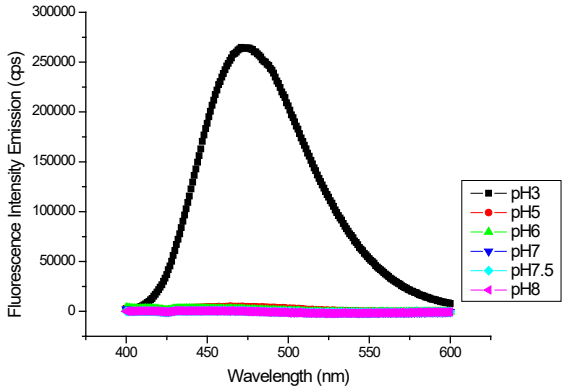

Figure 7. Extrinsic Fluorescence Spectra of Bio-GroEL at 10°C.

Bio-GroEL and GroEL were tested with a thermal melt increasing the temperature from 10°C to 87.5°C. These data were normalized with minimum-maximum normalization. The thermal melt also shows the structural alterations at pH 3. There is a transition apparent at pH 5, pH6, pH 7, pH 7.5, and pH 8. The transition takes place from 60°C to 70°C and remains structurally altered or aggregated to 87.5°C. GroEL and Bio-GroEL appear to have similar structurally regions and similar stable regions.

OD_{360} tests of Bio-GroEL and GroEL were conducted with a thermal melt increasing the temperature from 10°C to 87.5°C. During these tests the wavelength 360 nm was monitored. GroEL appears to become aggregated at pH 5 and pH 6. At pH 5 GroEL begins to aggregate at approximately 60°C, and at pH 6 GroEL appears to begin to aggregate at approximately 65°C. Bio-GroEL appears to begin to aggregate at pH 5 and pH 6 as well. At pH 5 Bio-GroEL appears to begin to aggregate at approximately 50°C, and at pH 6 appears to begin to aggregate at pH 70°C. GroEL and Bio-GroEL appear to have similar stable regions with the exception of the beginning of aggregation in pH 5 and pH 6.

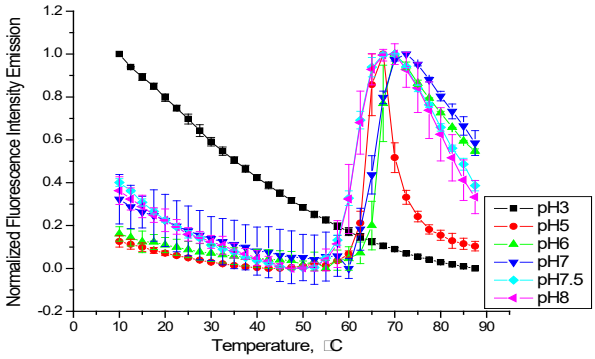

Figure 8. Extrinsic Fluorescence Thermal Melt of GroEL from 10°C to 87.5°C.

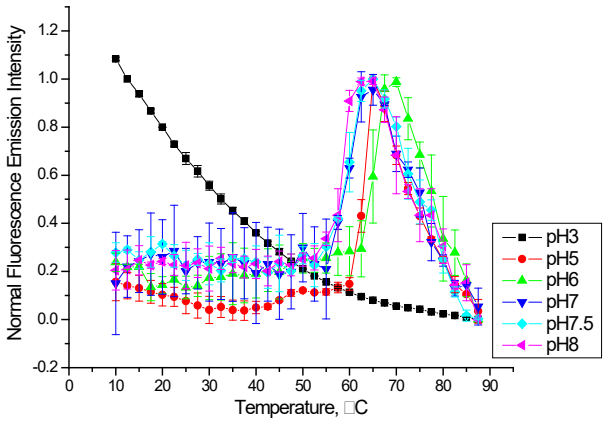

Figure 9. Extrinsic Fluorescence Thermal Melt of Bio-GroEL from 10°C to 87.5°C.

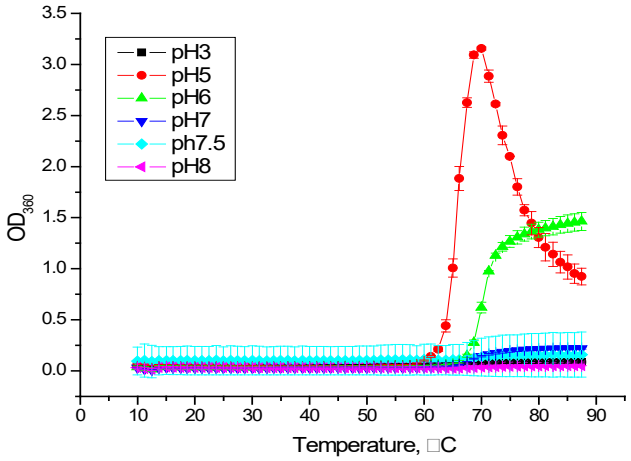

Figure 10. OD_{360} Thermal Melt of GroEL from 10°C to 87.5°C.

An empirical phase diagram (EPD) is a method used to incorporate all the biophysical data collected to identify parameter regions that define protein structural states as a function of solution variables. The data from the circular dichroism thermal melt at 222 nm, the extrinsic fluorescence with ANS, and the OD_{360} were incorporated into the EPD. Red represents circular dichroism, green represents extrinsic fluorescence, and blue represents OD_{360}. In the EPD's the roman numerals represent the different protein structural states: I represents the native state, II represents the structurally altered state, III represents the molten globule state, and IV represents the aggregated state. The native state in both GroEL and Bio-GroEL is from pH 5 to pH 8 and up to 60°C. The structurally altered state is present at pH 3. GroEL and Bio-GroEL both contain a molten globule state from 60°C to 65°C from pH 5 to pH 8. The aggregated state is present from pH 5 to pH 8 above 65°C. GroEL and Bio-GroEL appear to have very similar native and altered states.

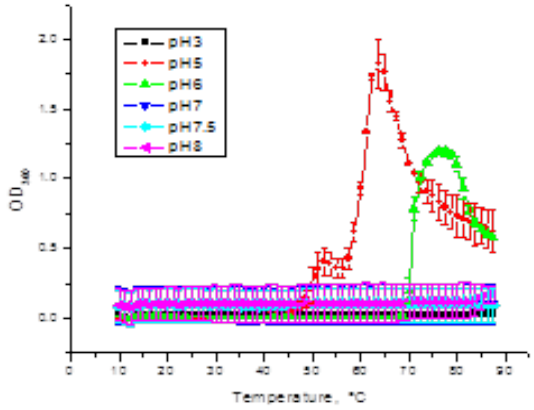

Figure 11. OD_{360} Thermal Melt of Bio-GroEL from 10°C to 87.5°C.

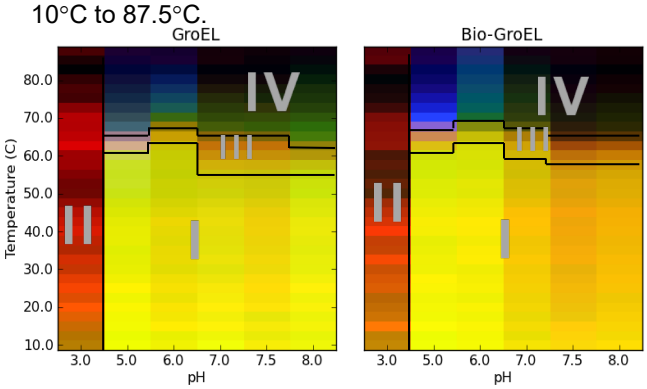

Figure 12. Empirical PhaseDiagram of GroEL and Bio-GroEL.

DISCUSSION

By using a variety of spectroscopic measures, GroEL and Biotinylated GroEL were characterized over a wide range of temperature and pH. GroEL and Bio-GroEL both exhibited structural changes in the aggregation state and in the secondary and tertiary levels. The stability of GroEL and Bio-GroEL was evaluated with an empirical phase diagram (EPD) technique. The empirical phase diagram technique is necessary to examine the data because the spectroscopic characterization of proteins generates large sets of data that are very difficult to interpret (Ausar, et al., 2006).

The EPD generated with the biophysical data shows the GroEL has a native state in the neutral pH's (5-8) and up to approximately 60°C. There is also a structurally altered state at pH 3. A molten globule state was observed from 60°C to 65°C and from pH 5 to pH 8. The molten globule state is when there is a native secondary structure intact with a structurally altered tertiary structure that admits solvent and lacks close packing (Baldwin, et al., 2012). The other state noticed is an aggregated state from pH 5 to pH 8 above 65°C. Aggregation can

take place by many mechanisms, and is classified in a number of ways, soluble/insoluble, covalent/noncovalent, reversible/irreversible, and natural/denatured (Cromwell, Hilario, and Jacobson, 2006). The transition states of Biotinylated GroEL are similar to those of GroEL. The native state is observed from pH 5 to pH 8 up to approximately 60°C. The structurally altered state is observed at pH 3, which is the same as GroEL. There is a molten globule state observed from 60°C to 65°C and from pH 5 to pH 8. The aggregated phase is observed from pH 5 to pH 8 above 65°C. Both GroEL and Biotinylated GroEL appear to be most stable at pH 5 and pH 6 up to approximately 62.5°C.

The circular dichroism (CD) data first suggests the structurally integrity of the alpha helices of the secondary structure are upheld from pH 5 to pH 8 but are compromised at pH 3 in both GroEL and Biotinylated GroEL (Figures 2 and 3). It also appears a slight pH dependency could be present due to the decrease in molar ellipticity as pH increases. The pH dependency is more obvious in GroEL than Bio-GroEL. Further research would have to take place to gain stronger evidence. When exposed to a thermal treatment from 10°C to 87.5°C there appeared to be a transition from native to structurally altered states which began at approximately 60°C and ended at approximately 70°C (Figure 4 and Figure 5). This is true for every pH except pH 3, it does not show a transition, which suggests pH 3 is structurally altered before the thermal treatment began.

Fluorescence spectroscopy typically excite a tryptophan molecule, but GroEL does not have any tryptophan so an extrinsic dye, ANS, was excited to run tests. As the protein unfolds ANS binds to exposed hydrophobic regions, which give a large signal. The fluorescence spectrum at 10°C shows the tertiary structure of GroEL and Biotinylated GroEL at pH 3 is altered and the remainders appear to be stable (Figure 6 and Figure 7). When exposed to a thermal treatment GroEL and Biotinylated GroEL at pH 5-pH 8 appear to begin to become structurally altered at approximately 62.5°C and are completely structurally altered by 72.5°C (Figure 8 and Figure 9). The difference between the beginning of structural alteration in the secondary and tertiary structure could be an explanation for the apparent molten globule state.

Using OD_{360} the aggregation state of the protein was examined. The wavelength 360 nm was observed because proteins do not absorb at 360 nm, so any observed absorbance would be caused by aggregation. In both Biotinylated GroEL and GroEL the onset of aggregation was at a lower temperature with a more prominent peak at pH 5 and pH 6 (Figure 10 and Figure 11). The other tests show aggregation with much less absorbance at approximately 70°C compared to the 60°C onset with pH 5.

The stability of GroEL and Biotinylated GroEL appears to be dependent on both temperature and pH. Low pHs such as, pH 3 and pH 4 cause both proteins to become structurally altered before a thermal melt. After 60°C the structural integrity of all samples was compromised. The stability of the proteins is important due to the fact that if they were to be used in a protein drug, if even a small percentage of the protein aggregates within the delivery system, serious safety issues such as adverse immunological responses could occur (Jiskoot, et al., 2012). If used in protein drugs varying ranges of temperature and pH are present in physiological conditions and the drug would need to stay stable through these varying conditions.

Biotin labeling was thought to possibly effect the physical stability of GroEL, because there have been many instances when commercially available biotinylation reagents may not be stable in biological fluids (Elia, 2008). The biotinylating reagents are rapidly cleaved from protein in plasma by the hydrazone bond formed between biotin-LC-hydrazide and carbohydrates present in proteins (Bogusiewicz, Mock, and Mock, 2004). The primary site of cleavage is between the carboxyl group of biotin and the ε-amino group of the lysine residue present in GroEL (Bogusiewicz, et al., 2005). Biotin labeling did not seem to affect the stability of GroEL: this could be in part that the experiment was done *in vitro* and biological fluids were not involved. Another reason is that the size of the GroEL molecule, 802 kDa, was much greater than the size of the biotin label, 556 Da. The similarity between the molecular weight of GroEL before and after biotinylation is visible in the SDS-PAGE. The Sulfo-NHS-LC-biotin kit used increases spacer arm link by arranging two aliphatic aminocaproic acid chains head to tail between the biotin and the sulfo-NHS group (Elia, 2008). The nature of the LC liner although soluble in water will be hydrophobic with this arrangement, which causes the biotin to seek hydrophobic regions in the protein (Elia, 2008). This causes an increase in stability as opposed to other biotin labeling methods.

With the knowledge that biotin labeling does not seem to affect the stability of GroEL *in vitro*, there are many possibilities for GroEL using biotin in the future. By labeling GroEL with biotin, many techniques can take advantage of the biotin-avidin interaction, which is considered the strongest noncovalent biological interaction known (Holmberg, et al., 2005). Biotinylated molecules can easily be detected using streptavidin derivatives or efficiently captured on avidin/streptavidin-coated solid supports (Elia, 2008). The problem with this bond is that the biotin-streptavidin complex is so stable that elution of the biotinylated molecules has proven to be difficult (Elia, 2008). The elution has been made possible by the discovery that dissociation is efficiently hindered with

both monovalent and divalent salts, which suggests the interaction is stabilized with ionic molecules, and that depletion of salts allows the complex to be dissociated (Holmberg, et al., 2005). Since it does not appear that biotinylation affects the stability of the chaperone protein, GroEL, research could take place to utilize GroEL in protein drugs in the future.

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