Using FCS to Explore Agglomeration States of Small Heat Shock Proteins

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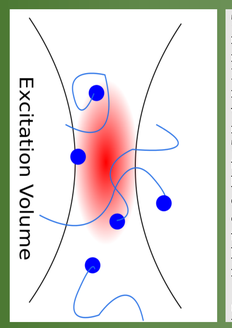
Abstract:

Small heat shock proteins (sHSPs) are found in nearly every form of life. These proteins assemble in oligomeric cages of various sizes and shapes, and they function as chaperones, protecting the cell from protein aggregation and targeting other proteins for degradation. However, the exact mechanism of this chaperone activity has not been discovered. One hypothesis says sHSPs are only active when broken apart into smaller subunits like dimers, while others say the oligomeric form of sHSP also plays a key role in protein stabilization. One example of an sHSP is Mj HSP16.5 which comes from *Methanocaldococcus jannaschii*. This organism is an archaebacterium, which is thermophilic and methanogenic. Mj HSP16.5 is usually found as an icositetramer, which is highly resistant to heat. Using dual excitation fluorescence correlation spectroscopy (FCS), we explore the conditions required for subunit exchange in Mj HSP 16.5. Our results suggest under harsher conditions (high temperature and low pH), more subunit exchanging occurs, which is indicative of sHSPs breaking in smaller oligomers.

Introduction:

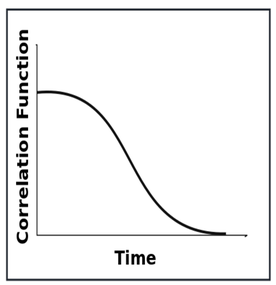
sHSPS are part of the molecular chaperone family (1). These proteins can prevent the detrimental aggregation of nonnative proteins when cells become stressed, and they are highly conserved among organisms (1). It is known that sHSPs have chaperone-like properties, meaning they help protect and fold other proteins, especially when cells come under stress from temperature or pH (1). Malfunctioning of sHSPs can result in cancer, cataracts, and neurodegenerative diseases (1). It is also known that sHSPs can take a monomeric or an oligomeric form and that the aggregation and separation of these oligomers are controlled by environmental factors such as temperature and pH, but there are questions about the specifics of this process. It is also unclear how the aggregation state relates to chaperone activity. One hypothesis is that the oligomeric forms of sHSPs are simply for storage and that smaller subunits are more active as chaperones, but this has not been confirmed (1).

Some questions about sHSPs can be explored using fluorescence correlation spectroscopy (FCS). FCS reveals information about molecular structure, kinetics, and dynamics by measuring how fluorescence changes from moment to moment in a dilute sample (2). Fluorescence intensity changes when molecules diffuse into or out of the laser focus. The width of the correlation function depends on how fast molecules move through the focus, while the height of the correlation function depends on the number of molecules in the focus at a given time. See images below. In our case, we anticipate that a single sHSP, or a monomer, will diffuse through the laser focus faster than 2 or 4 or 10 or 24 sHSPs agglomerated together. We also anticipate that if the sHSPs are in a smaller oligomer, we would see a much greater number of species present in our sample. In other words, the height and width of FCS correlation functions could give valuable information about the state of sHSPs – exploring these states is the long-term goal of this project. In addition to molecule size, FCS curves are sensitive to changes within molecules. For instance, energy transfer between two dyes on the same complex (FRET) will change the height and width of correlation functions (3).



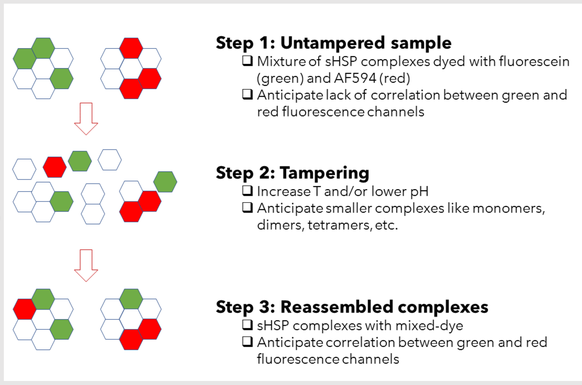
The image on the left illustrates molecules moving through a microscope focal volume. Fluorescence fluctuates as the number of molecules in the focal volume changes from moment to moment.
The image on the right shows a typical correlation function. The height of the correlation function depends on the average number of molecules in the sample, while the width of the curve reflects how long it takes molecules to move through the focus.

(Images from McMaster University)



Experiment:

In this poster, we report on initial experiments that use FCS measurements to observe sHSP samples before and after being exposed to conditions that might alter their oligomeric state. The general workflow is shown to the right. We started with untampered samples that were mixtures of fluorescein and Alexa Fluor 594 labeled sHSPs. We then subjected these mixtures to various harsh conditions. These conditions were a control, heating up to 65°C, heating up to 85°C, lowering the pH to 3, and lowering the pH to 3 while heating at 65°C or 50°C. Following these tampering steps, we brought the samples back to room temperature and/or pH 7.4. We performed two-color FCS measurements on both the untampered and reassembled samples. The results are reported below and to the right.



Results:

FCS curves for several perturbing conditions are shown below, and all our data is summarized in the table to the right. Collectively, this data suggests the following:

- We observed no evidence of subunit exchange for either the control (untampered) samples or the samples perturbed at 65°C. Correlation functions were largely unchanged by this tampering, and FRET efficiencies were low.
- We observed some evidence of subunit exchange for samples perturbed by very high temperatures (85°C) or low pH.
- We observed strong evidence of subunit exchange for samples perturbed by both high temperatures and low pH. Most notably, the largest increase in FRET efficiency was observed with lowering the pH to 3 and heating at 65°C or 50°C.

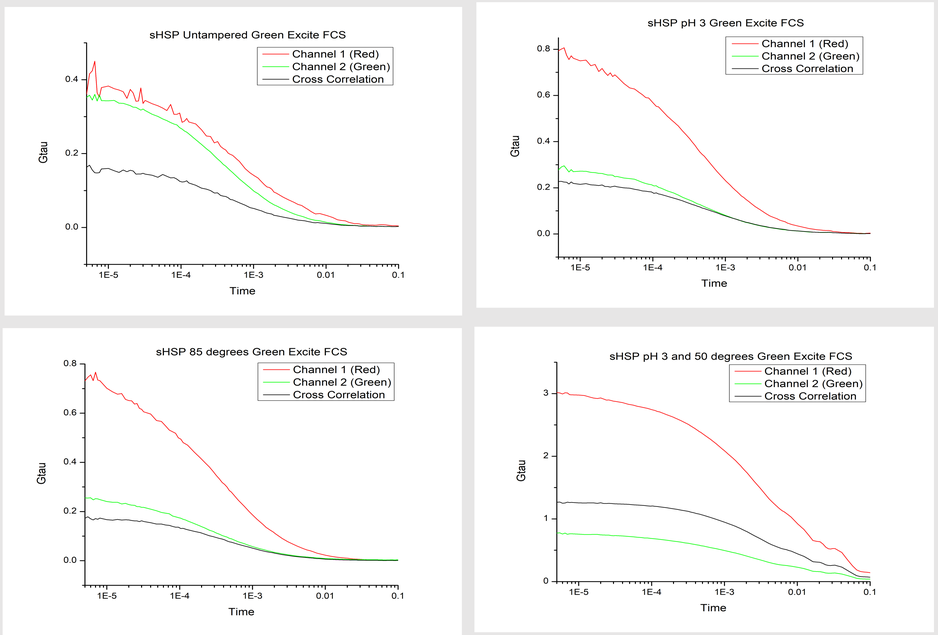


Figure 1: (Top left) FCS curves for channel 1 (Red), channel 2 (green), and cross-correlation based solely upon green excitation of untampered sHSP. The similarity of the red and green curves may indicate bleed through is having a large impact on channel 1. (Top right) FCS curves for channel 1 (Red), channel 2 (green), and cross-correlation based solely upon green excitation of sHSP exposed to pH 3 for 30 minutes at room temperature. (Bottom left) FCS curves for channel 1 (Red), channel 2 (green), and cross-correlation based solely upon green excitation of sHSP exposed to 85°C for 10 minutes. (Bottom right) FCS curves for channel 1 (Red), channel 2 (green), and cross-correlation based solely upon green excitation of sHSP exposed to 50°C and pH 3 for 10 minutes.

Disturbance	Gred (ch1)	Ggreen (ch2)	Gcross	% Green (488)	% FRET Efficiency
Untampered	.358 / .383	.355 / .359	.162 / .028	82	9.9
Untampered (free dye mix)	0.057 / 0.034	0.071 / 0.069	0.033 / 0.036	91	0
Untampered (fluorescein only)	0.16 / 0.05	0.09 / 0.09	0.07 / 0.06	93	NA
pH = 3 for 30 minutes @ RT	.805 / .494	.275 / .279	.227 / .133	70	23
pH = 3 and 50°C	2.90 / 2.09	0.71 / 0.84	1.15 / 1.02	66	27
pH = 3 and 65°C	7.78 / 6.09	1.80 / 1.84	3.08 / 2.71	57	37
85°C	0.73 / 0.53	0.26 / 0.25	0.17 / 0.13	68	25
65°C	0.49 / 0.37	0.27 / 0.27	0.13 / 0.029	80	12

Table 1: The first three columns compare the Gtau at 5 microseconds for 488 nm excitation / Gtau at 5 microseconds for total excitation (488 nm excitation plus 594 nm excitation). A lower Gtau in the first two columns indicates more fluorescent species. A higher Gtau in the third column indicates a greater cross-correlation between the green and red species. The fourth column gives the percent of photon counts which hit the green detector after green excitation. The final column shows the percentage of photons that show up in the red channel after green excitation while adjusting for bleed-through and direct excitation of AF594.

Discussion:

To assess whether subunit exchange was occurring, we looked at Gtau values at 5 microseconds for 488 nm excitation and Gtau values at 5 microseconds for total excitation (Table 1). We also looked at the percentage of photon counts which hit the green detector from green excitation and FRET efficiency (Table 1). From this data, we saw some subunit exchange occurs at temperatures of 85 degrees *or* pH levels of 3, but more subunit exchange occurs at pH levels of 3 *combined with* temperatures of 50°C and 65°C (Table 1). One possible explanation for this is, in harsher conditions, sHSPs break into smaller subunits so they can better stabilize proteins. This scenario would result in more diverse agglomeration of our two species and a greater FRET efficiency, which is precisely what we see in the harsher conditions. One further direction for this research is to get dyes that are less pH-sensitive and temperature-sensitive so we can observe sHSPs as smaller oligomers. This would allow us to look at Tau diffusion times and determine the relative size of the subunits. Another direction would be making a scatterplot of various temperature and pH values and the level of cross-correlation. This scatterplot would also provide insight into the size of subunits by looking at the degree of subunit exchange.

Materials and Methods:

Overexpression: We were gifted a DH5alpha agar stab from Addgene that contained the plasmid, pET21a.MJ0285 from Sung-Hou Kim (Addgene plasmid # 11304; http://n2t.net/addgene:11304; RRID:Addgene_11304), which encodes small heat shock protein from *Methanocaldococcus jannaschii*. The plasmid contains an ampicillin (Amp) resistant marker, so we plated the DH5alpha onto an LB 2X Amp plate. These colonies were grown overnight at 37°C. We picked four of the colonies and grew them overnight in 5mL LB with 1X Amp at 37°C. Then we did a miniprep following the Monarch Plasmid MiniPrep Protocol (New England Biolabs, Ipswich, MA). We got a DNA yield of 42.2 ng/µL, according to nanodrop analysis. This DNA was used to transform into Rosetta™ 2(DE3)pLysS Singles™ Competent Cells – Novagen (EMD Millipore Corp., Burlington, MA), according to their user protocol. This cell line was chosen because Rosetta 2 expresses rare codons which are found in some archaea, like *M. jannaschii*. Four starter cultures were made using different colonies from the Rosetta 2 plate, and they were grown overnight. We made four liters of culture using 5 mL of starter culture, 1X Amp, 1X Chl, and 1 L of TB-AIM. These cultures were grown for 48 hours at 37°C before pelleting at 7,500 rcf for 15 minutes.

Purification: Purification consisted of resuspending a bacterial pellet in 10 mL of 100 mM HEPES at pH 8 and 1X Protease Inhibitor Cocktail (APExBIO, Boston, MA). The pellet was homogenized and kept at room temperature for 30 minutes. We did 3 rounds of sonication on ice, with the settings 2 seconds on, 6 seconds off, for 5 minutes, and a 3-minute rest period between rounds. We set the Branson SFX 150 Sonifier (Brookfield, CT) to 70% power. This mixture was centrifuged for 70 minutes at 10,000 rcf, and the supernatant was decanted. To the supernatant, we added Ribonuclease A from bovine pancreas (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10 µg/mL, Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1 µg/mL, $MgCl_2$ to a final concentration of 20 mM, and $CaCl_2$ to a final concentration of 10 mM. Salts were added for optimal DNase I function. The mixture was placed in a 60°C-water bath for 30 minutes. The sample was then kept on ice for 10 minutes before being centrifuged for 80 minutes at 10,000 rcf and filtered through a 0.2 µm PES filter. 5 mL of this mixture was injected into the FPLC and allowed to flow through a size exclusion chromatography column with the 100 mM HEPES pH 8 buffer flowing at a rate of 0.2 mL/min. We then ran SDS-PAGE to determine which peak fractions had significant levels of protein that ran to 16.5 kDa because this is the monomeric mass of sHSP.

Labeling: We labeled a sample of purified sHSPs with 5(6)-SFX (Fluorescein), SE (Tocris, Bristol, UK) following the manufacturer's conjugation protocol for amine-reactive dyes. We labeled a different sample with Alexa Fluor™ 594 (AF594) NHS Ester (Succinimidyl Ester) (Thermo Fisher Scientific, Waltham, MA), following their amine-reactive probes protocol. We used an FPLC (akta-pure) equipped with two HiTrap Desalting columns (Cytiva, Marlborough, MA) to separate the free dye from the protein.

FCS Measurements: In this study, we utilized a confocal microscope and avalanche photodiodes from ISS (Champaign, IL). The wells on the CELLview™ Cell Culture Slides (Greiner Bio-One, Monroe, NC) were incubated with 10 mg/mL BSA to ensure that proteins would not stick to the slide. We used dual excitation at 488 nm and 594 nm with power levels around 1 µW, for each wavelength when measured immediately before the laser entered the microscope. We then took 10-minute FCS measurements of mixtures of fluorescein labeled and AF594 labeled sHSP that had been subjected to various conditions. These conditions included a control, heating to 65°C, heating to 85°C, lowering to pH 3, and lowering to pH 3 while heating tp 65°C or 50°C. In each case, after 10 minutes of the condition (30 minutes for pH 3), we would bring the sample back to room temperature and pH 7.4, which allows the dye to function normally.

References and Acknowledgements:

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