Haverford College

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

Haverford College received $28,802 in formula funds for the grant award period January 1, 2012 through June 30, 2015. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Mapping Influenza Hemagglutinin Proteins by Raman Spectroscopy – Hemagglutinin (HA) is one of the two main surface proteins on the influenza virus, and changes in its shape and amino acid sequence have been associated with increased virulence in strains like the 1918 "Spanish flu" and the 2009 "swine flu" outbreaks. The aim of this project is to use epsilon-deuterated histidine (His) as a site-specific probe of each of the surface histidine residues in HA to report both their protonation states and their level of solution exposure. Using Raman spectroscopy of C-D vibrations located on surface His residues as a novel spectral approach, we will establish a spectral map of the HA surface and then observe how the environment of the His residues changes across different strains.

Anticipated Duration of Project

1/1/2012 – 6/30/2015

Project Overview

Histidine (His) side chains in proteins have unique, pH-dependent properties that can mediate assembly and function of large biomolecular assemblies, including the assembly and maturation of viral particles from their constituent parts and the maturation of these particles into infective agents. There is no good replacement amino acid for His, and the most typical site-specific technique used to examine the functional structure and interactions of His residues is nuclear magnetic resonance (NMR) spectroscopy, which is severely limited both in its ability to detect functional dynamics at His and in its solution-phase limitation to small, globular proteins. The epsilon hydrogen of solution-exposed His residues can be exchanged for deuterium under relatively mild solution conditions, leading to a unique and strong chromophore for Raman spectroscopy.

Raman spectroscopy has no limitations on the size or phase of the system of interest. In this project we will attempt to characterize the clusters of surface His residues on the influenza protein hemagglutinin (HA), a viral surface receptor and the main target for the immune

response to the flu virus. With the high level of glycosylation in human HA and the protein's pH-directed intracellular maturation and assembly pathway, it is likely that different clusters of His along assembled HA will exchange their epsilon protons for deuterium at different rates. It is also known that commercially available antibodies bind tightly to different regions of the HA stalk structure. Between varying exchange rates and selective antibody binding (and resorting to mutagenesis if necessary), we will constitute a spectral map of all of the HA surface histidines by observing the C-D(epsilon) Raman peaks from selected groups of His. We will then use this spectral map to investigate variations in the structural flexibility and pH-mediated structural changes of HA from flu strains of varying virulence, with the possibility of validating a current bioinformatic hypothesis that structural flexibility in selected regions of HA confers greater virulence.

Aim 1: Epsilon-deuterated histidine will be established as a method to interrogate the surface histidine residues of hemagglutinin (HA) proteins.

a. The sensitivity of the C-D stretching band from His(epsilon-D) will be established using model compounds, and the detection limits (in molar concentration of the probe group) will also be clarified.
b. HA from the 2009 H1N1 influenza strain will be expressed and purified from human kidney cells and subjected to conditions that facilitate the deuterium exchange of the His epsilon protons.
c. C-D stretching Raman signals will be collected from the 2009 H1N1 strain at varying deuterium exchange times to determine the spectral response of His that exchange at different rates.

Aim 2: The spectral response of particular groups of His will be used to develop a spectral map of His in the structure of the 2009 H1.

a. The mapping strategy will include data from exchange rates (aim 1c), pH-dependent Raman spectra, and the spectral response to region-specific binding of HA antibodies, with the goal of elucidating what is likely to be many overlapped signals in the Raman spectrum.
b. In the absence of a clear map from aim 2a, site-directed mutagenesis to replace specific His residues will be used to locate their contributions to the Raman spectrum.

Aim 3: Using the spectral map from 2009 H1 as a starting point, the responses of His from other HA strains (including 1918 H1 and several less virulent HA sequences) will be compared.

a. New strains will be subjected to an expedited version of Aim 2 so that their Raman spectra are clear with respect to specific His residues.
b. Differences in the spectral response of particular His will be compared to bioinformatic predictions about the variable flexibility and structural disorder in particular structural regions.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

The anticipated outcomes of this work are twofold: 1) the establishment of a new spectroscopic method to interrogate large viral proteins, and 2) an enhanced understanding of the relationship between structure and virulence in influenza hemagglutinin proteins.

Current approaches to the dynamic characterization of large viral protein assemblies are quite limited: most structural information comes from crystal structures of proteins whose native glycosylation patterns have been edited to assist crystallization. Raman spectroscopy provides both a much faster dynamic window and a greater sampling flexibility than almost any other spectroscopic technique. The unique C-D(epsilon) chromomophore on His, and the high variability of this vibrational frequency depending on its environment, can provide a site-specific viewpoint otherwise absent from protein vibrational spectra. A spectral map of surface His residues in HA would be a major step forward in establishing this technique for evaluating the interactions and dynamics at the surface of large viral protein assemblies.

Directly observing flexibility differences, or other dynamic changes, across HA proteins from different flu strains would validate or negate the current hypothesis that enhanced flexibility in certain structural regions of HA leads to increased virulence. If this hypothesis is validated, it would clear the way for flexibility-based analyses (both *in silico* and *in vitro*) to predict the virulence of new flu strains, as well as to establish abrogation of site-specific flexibility as a strategy for combating particularly difficult flu strains.

Summary of Research Completed

Progress was difficult during this reporting period, mainly due to two ongoing problems: facilities issues and continuing personnel turnover. Further progress was made towards aim (1) but aims (2) and (3) remain delayed due to challenges associated with making and purifying enough sample. This led to a temporary change in scope, which proceeds along similar lines and might have similar outcomes, but uses slightly different methodology and a different viral system to achieve some of the same goals. The grant was extended until 2015 in order that we might be able to make further progress towards aims (2) and (3) with personnel trained in summer 2014.

Further progress towards aim (1) involved working with a slightly different model compound,

the free amino acid *l*-histidine-*d*2, in solutions of varied pH to determine a very clear lower detection limit for the C2-D vibration of interest in our Raman spectrometer. We really did everything possible during this grant period to increase the sensitivity of our home-built instrument, short of completely changing the optical layout and adding major new components that would be beyond the scope of the funding available here. We are now currently capable of interrogating samples that have a volume of 1 microliter, and we have established a clear lower detection limit for the C2-D vibration of l-histidine-*d*2 our instrument of 1 mM. This appears to be a relatively hard and fast lower limit using non-resonant excitation sources; moving to an excitation wavelength of 488 nm (from 514 nm) offered a nominal benefit (about 20% at the same power densities) without dramatically increasing our sensitivity. We tried increasing the incident power at the sample to 250 mW at both laser lines and decreased our effective collection time for a good Raman spectrum at higher concentrations, but 1 mM samples still need multiple hours to build up enough signal to see a single His residue at this concentration, which is still relatively high for large protein complexes.

Our efforts to isolate enough HA to work with in multiple deuterium exchange experiments are as yet unsuccessful. We did change our protocol to work at larger volumes of HEK293 cells in spinner flasks, and we successfully transfected a 1 L culture of these cells in a spinner flask with the HA plasmid from the Nabel lab with what appeared to be good results according to microscopy of the cells, whose morphologies and adherence to each other changed greatly due to the expression of HA proteins which are trafficked mainly to the cell surface. We centrifuged these cells and filtered the supernatant, and then isolated only a small but measurable amount (about 0.002 mg) of aqueous HA by His-tag affinity chromatography. According to Western blotting, the filtering step is where we lost most of the product, which appears to stay with the viscous post-cellular products and elude the filtrate. We are set up to do this again without the filtering step, at which point we would expect a 1-5 mg/L yield of HA based on previous work from a few other groups. However, in all previous cases, we have noted that large-scale production of purified HA is generally quite challenging and we may be trying to make more of this purified protein assembly in one place than has ever been made before using recombinant methods.

The spinner flask treatment was conducted by a new student, who took up the project following the work of two previous students, one of whom stayed on as a technician. That student has now graduated and a fourth student is being trained to use the Raman spectrometer and to conduct the cell culture experiments necessary to produce these samples, and that student will be the main worker on this project through the end of the grant in summer 2015. We have all of the steps in place to produce enough protein, but unfortunately our Biosafety Level 2 (BSL-2) certified collaborator in the Biology department left Haverford (officially during 13-14) and the Biology department terminated all BSL-2 certified activity in their department on her departure. To work with HEK 293 cells requires BSL-2 certified facilities. The Biology department has kindly provided a space that can be used for BSL-2 activities through summer 2015, but that space has not yet been certified and the new student has not yet been trained in BSL-2 protocol yet. Our hope is that this will be running in earnest by fall 2014 and that we can spend a few weeks in earnest making and isolating enough sample for our desired deuterium exchange experiments.

The sample requirements for HA in our spectrometer will be fairly steep; although we can look

at very small samples, a 1 microliter sample would still require about 0.1 mg of protein per sample, and effectively closer to 1 mg per sample given the volume requirements of standard centrifugal concentrators. Our solid detection limit indicates that to identify single His residues in HA trimers, we really should work at above 400 μM concentration for each spectroscopic sample. These samples will provide a difficult test of our instrument.

With that sample-related challenge in mind, we have begun a new collaboration on another protein associated with the flu virus that possibly presents a somewhat simpler preliminary step towards applying this methodology to viruses. This different direction applies the C2-D vibration of 2-deuterated histidine to the His37 residue of the influenza M2 proton channel, which is necessary for viral assembly and maturation. His37 has long been implicated in this more-studied membrane protein system as a pH-sensitive trigger for proton pumping, but the exact protonation state and interactions between multiple His37's has not been directly measured by any technique to this date. We endeavor to observe the His37 C2-D vibrations in the transmembrane domain of the M2 by creating labeled peptides that will self-assemble into functional M2 channels following extensive prior work by William DeGrado, Mei Hong, and many others.

The DeGrado lab (at University of California, San Francisco) has kindly offered to synthesize the peptide for us, and we have provided the protected and labeled histidine amino acid for incorporation during that synthesis. To produce this synthetic precursor in high yield, we first 2-deuterated l-histidine following standard lab procedure, then performed consecutive organic synthesis reactions to add first, a trityl protecting group to the imidazole ring and second, a fmoc protecting group to the free amine so that the amino acid could be incorporated using standard fmoc-protected peptide synthesis chemistry (following the DeGrado lab's specialized protocol for hydrophobic peptides). At the date of report submission, an initial synthesis of this peptide has been successful and the peptide is being purified for Raman spectroscopy experiments. The additional synthetic methodology used here yielded a large amount of protected and labeled histidine which might be used for other peptides in the future that might be less amenable to post-translational labeling than large, soluble assemblies like HA, and it provides a strong basis for extension of this methodology to other peptidic and membrane-based species.

Constitution of these samples for Raman spectroscopy will involve mixing the peptide with either detergent or lipids and sonication to insert the peptides into the micelles or bilayers. It is known that the M2 transmembrane tetramer channel can be assembled successfully at concentrations up to 10 mM of peptide in DPC micelles, so we are confident that we should be able to see signals quickly from these samples. The results of even our initial experiments might answer a long-debated question in virology and flu research in this otherwise well-characterized protein system, and would provide a strong case for the possible extension of our methodology to other viral problems.