Femtosecond Studies of Protein Hydration

Margaret K. Penner

Department of Physics
Bethel College
North Newton, KS 67117

The Ohio State University
Physics REU Summer 2004
Advisor: Dr. Dongping Zhong

August 20, 2004
Abstract

This summer we used pump-probe experiments to determine the time-scales of hydration in apomyoglobin. To do this we mutated the protein to include Tryptophan as a strategically-placed optical probe, and used a fluorescence upconversion technique to measure the intensity of fluorescence with respect to time at various wavelengths. Additionally we examined the rotation of the protein and the effects of varying the pH of the environment. Once analyzed, our data should contribute to the knowledge of exactly how changes in the structure of proteins occur.

I. Introduction

In the past several decades, scientists have applied the methods and theory of physics to the study of chemical processes. In doing so, they more or less elucidated the molecular structures of the chemical systems before and after the processes. Now, as these same methods are being used to study biological systems like proteins, the question of how fast these reactions happen becomes more important. With a method invented in the late 1980s by Professor Ahmed H. Zewail of California Institute of Technology, we are able to determine these timescales and gain a much deeper understanding of the systems which we study. This summer we applied such a method to a specific protein dissolved in water in order to determine how fast the surface water moves.

II. Background

Protein function and stability are determined solely by structure (primary, secondary, tertiary, etc.), and in nature, proteins are almost always found dissolved in
water. Indeed, without water, proteins are physiologically inactive. Thus, the more we know about protein structure and how that structure changes (i.e. protein dynamics) in the presence such solvents, the more we know about the function and stability of the protein. This knowledge greatly contributes to the basic understanding of biological systems, which is our ultimate goal. This summer our research has been centered around determining the timescales for the movement of water around apomyoglobin.

We use an optical probe to aid us in our study, usually Tryptophan (Trp) because we know so much about its optical properties and because its optical properties are so strongly related to its environment. We mutate one strategically placed amino acid in the protein into Trp (see fig. 1). That is the portion of the protein which we wish to study in depth, because when Trp is in close proximity to water, some of its excitation energy goes into dipole-dipole interactions, decreasing the energy bandgap, and shifting its emission spectrum. This is known as a Stokes shift. By looking at trends of this in solutions of a protein in buffers at various pH values and at different wavelengths and polarizations, and using our knowledge of Trp’s normal optical properties as well as the usual structure of the protein in question, we can determine the behavior of the protein in that particular environment.

Since one pH value corresponds to the native state of the protein, we also vary the pH values at which we take data in order to investigate the water dynamics on the protein surface in the native and non-native states of the protein. For the apomyoglobin mutants we used, the native pH is 6.4; we took data at pH=6.0 as well as at pH=4.0 which is the molten-globule state, the critical state where approximately half the protein is unfolded.
III. Methods

III. 1. Protein Mutation

In order to study protein dynamics, we must first obtain protein with the specific properties that we wish to study with appropriate optical markers in place. Our group makes these proteins through site-directed mutagenesis and prepares the protein for study through various stages of purification.

The goal of site-directed mutagenesis is to change one specific amino acid. To do this, we must change one group of three bases on a strand of DNA, which is done through PCR (Polymerase Chain Reaction). Using natural DNA as a template (for example, for myoglobin from the muscle cells of a sperm whale), we order two mutagenic primers - one for the top and one for the bottom - from a company. These primers must be complimentary, which means that their bases are in such an order that A is always opposite T and C always opposite G. The PCR is done by mixing the primers with the DNA, as well as some buffer, dNTP, DNA polymerase, and distilled, deionized water. This solution is subjected to a series of cycles at various high temperatures to denature (essentially unravel) the DNA, anneal the primer to the template DNA, and subsequently allow the DNA polymerase to elongate and form the compliment to the template.

To verify the success of mutagenesis, we transform this DNA into XL1-Blue cells: We spread these cells on an LB and Amp plate and leave at 37°C overnight. Then we pick one colony for yet another overnight culture, this time in a larger test-tube with more medium and antibiotic. The next day we extract the DNA, isolating it by making a pellet of the cells, re-suspending the pellet, lysing the pellet by Alkali solution, neutralizing it
again, binding the DNA onto a column, washing it several times and finally eluting the DNA from the column once more. Then, after measuring the DNA’s absorbance and calculating its concentration for our records, we send it for sequence.

If the sequence that is returned matches the gene sequence we were trying to obtain, we can go ahead and grow the cell by transformation of the DNA again, this time into TB1 cells. As before, we grow it first overnight on a plate, but then pick one colony for overnight culture in 50 ml LB and from there we grow 4L overnight culture by diluting the 50 ml overnight culture 1:100. This whole process is known as protein expression. The gradual volume increase is necessary for good efficiency so that the LB can be saturated in a timely manner without causing cell deaths by over-saturation. The saturation is checked via optical density.

Once we have the desired protein in the cell, it only remains to be extracted from the cell and purified. First we spin it down in the centrifuge to obtain a pellet which is then frozen at -80°C for preservation, and at some later date we lyse the cell with different enzymes (lysozyme, RNaseA, DNase1) and sonicate it. These processes degrade the cell wall and digest DNA and RNA so we can obtain just the extract containing our interesting protein. Running this extract through a centrifuge allows us to separate the cell debris and unbroken cells from the protein. We then run the protein through several columns which separate it by molecular size and by charge (called gel filtration and ion exchange, respectively). By switching collection test tubes after a set volume has been collected, we maintain a separation between the fractions of the protein according to how large they are. We can test the purity of the protein in specific fractions after each run through a column by running a sample of it through SDS-PAGE.
(polyacrylamide gel electropholysis) which separates the protein by size again, showing us how pure each fraction is. Once the desired purity level is reached, the protein is stored at -80°C until it is used to take data.

Mutants are denoted by a syntax indicating what amino acid was originally present (abbreviated with one letter), on which site the mutation occurred (denoted with a number), and to what it was changed (abbreviated with one letter). For example T95W denotes the mutant in which the ninety-fifth amino acid was changed from threonine (T) to tryptophan (W). This summer we took data on apomyoglobin mutants T95W, H48W, A144W, G129W, K87W, and H113W (see fig. 1).

**Figure 1**

*The protein structure of apomyoglobin with each amino acid highlighted that was mutated to Trp in our research this summer. Note that each sample had only one of these mutations present.*
III. 2. Optical Setup

We obtain a sample of protein dissolved in buffer of a known pH from the bio lab, and pipette a small amount of this into a viewing cell between two thin quartz windows for viewing. This we place in rotating cell, ensuring the sample is not burned and no build-up occurs, while enabling us to focus a laser beam on it and collect its fluorescence. This is done using the set-up on the optical table (see Fig. 2).

![Optical Table Diagram]

**Fig. 2**

*The optical table.*

When we take data, we simply measure the intensity of the fluorescence of the sample at particular wavelengths and polarizations with respect to time after the sample is excited through a technique called fluorescence up-conversion. We use this information
to determine how fast various processes happen, such as hydration, solvation, desolvation, recognition, etc., as well as determining the structure of the protein through the proximity of our optical probe, Trp, to quenching agents.

To obtain a time-resolved measure of the fluorescence we use the “pump-probe” technique: We split our pulsed laser beam (800nm, 100 fs, 1 kHz) into two identical, synchronized beams, a pump beam to excite the sample and a probe beam to act as a gate. The pump beam travels through a series of optical devices including BBO crystals which modify its wavelength using non-linear optical methods, two prisms which compress the pulse, and a chopper (500 Hz) that blocks every other pulse, aiding the data acquisition system in determining the proper signal. It then is reflected by a retroreflector, two mirrors mounted perpendicularly on a translation stage, which can be controlled automatically by the data acquisition software to an accuracy of 30 µm, thus specifying the distance traversed by the beam and, more importantly, the time at which it arrives at the sample, which we shall discuss further below. After traveling through more non-linear crystals as well as several lenses that ensure that the pulse has the proper wavelength and is focused on the sample, the pump beam enters the sample, exciting it and causing it to fluoresce. This fluorescence is collected by a parabolic mirror that is confocal with the lens immediately preceding the sample and thus sends the fluorescence as columnated light to a second parabolic mirror, as the original pump pulse is blocked by a strategically-placed optical barrier. The second parabolic mirror focuses the fluorescence on another BBO crystal which mixes it with the corresponding probe beam pulse. This probe beam pulse has traveled a set distance through various optical devices which ensure its purity, focus, proper wavelength, and proper polarization.
Here we note that the aforementioned translation stage enables us to make the pump pulse excite the sample any desired point in time, so that the fluorescence mixed with the probe pulse is actually from a particular time after the sample’s excitation. This is how we can measure intensity of fluorescence as a function of time with such excellent resolution (in 100s of femtoseconds), which is not obtainable via direct electronic measurements.

The fluorescence is mixed with the probe beam via non-linear optic techniques that simply combine the energy of two photons, one from each light source, provided the beams meet the phase-matching conditions (including frequency and appropriate angles), and emits a photon with an energy that is their sum, which is how the fluorescence is up-converted. This is the signal that is read by the monochrometer, adjusted to collect the desired wavelength, for which the BBO-crystal is also properly angled, and passed on to the data acquisition system.

IV. Analysis and Results

The raw data that we obtain is simply a measure of intensity of fluorescence at a particular wavelength with respect to time. This data is normalized, has any Raman peak subtracted from it (see Fig. 3) and is then combined with other data sets from the same sample at different wavelengths to create a time-resolved emission spectrum (see Fig. 4 insert).
The emission spectra vary with time because of the Stokes shift. When an amino acid becomes excited it becomes slightly polarized. If free water is nearby, the water will interact with the polarized molecule by rotating as a result of a dipole-dipole interaction. In this way the excited molecule releases energy without radiating (fluorescing) and the energy bandgap is decreased. Thus when fluorescence does occur, the emission spectrum is red-shifted. As time after excitation goes on, this shift becomes increasingly evident because there is more time for the dipole-dipole interactions to occur.
Clearly from a collection of these curves we can determine the timescales of water movement on the protein’s surface, and we do this formally by constructing the hydration correlation function $c(t)$, a normalized plot of wavenumber vs. time, from the log-normal fittings of many time-resolved emission spectra (see Fig. 4). The biexponential fitting of $c(t)$ describes the motion of the water on the surface of the protein, and is completely independent of the optical probe used to collect the data. From the $c(t)$ given in Fig. 4 we can easily see that the first decay happens at approximately 2 ps and the second, slower decay at approximately 45 ps. These correspond to the two modes of water movement on the surface of the protein. By comparing the $c(t)$ for various proteins under different conditions as well as with the optical probe at different sites, we gain an
even clearer picture of what is happening on an ultrafast, molecular scale. We are still analyzing the results from this summer's data to understand the broader implications of our research.

We also gain some information about the rotation of the chromophore (Trp) by looking at the polarization of the fluorescence: Initially, the fluorescence has the same polarization as the pump pulse. Thus, if the pump and probe beams have parallel polarization, the signal should be very strong, and if they have perpendicular polarization, the signal should be the weakest. However, as time passes, the molecules tend to rotate, giving off light that has increasingly more non-parallel components of polarization. Thus, even if the lifetime of the fluorescence is quite long, if the pump and probe beams have parallel polarization, the signal will decrease at a certain rate, and if they have perpendicular polarization, the signal should increase at that same rate. This rate is a measure of the rigidity or viscosity of the protein, which we call the anisotropy. It is given by $r(t) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$. The magic angle is the angle between the polarizations of the pump and probe beams at which the signal should remain constant. It is given by $I_{\text{magic}} = \frac{I_{\parallel} + 2I_{\perp}}{3}$, and for all samples this angle is $54.7^\circ$.

V. Acknowledgements

I would like to thank Prof. Dongping Zhong and the Zhong group for graciously and patiently teaching me about biophysics, the National Science Foundation for their financial support, and my advisor, Prof. Don S. Lemons of Bethel College, for whole-heartedly encouraging me in my various pursuits of physics.
VI. References


