Structural Dynamics of Photoactive Yellow Protein

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Abstract

Photoactive Yellow Protein (PYP) is considered to be a model chromophoric protein. The dynamics of its active site during photoisomerization are considered to be a novel effect which have been looked at by several research teams around the world. Our research group designed a new time-resolved experimental system using a tunable laser and Fourier-Transform Infrared (FTIR) system in tandem. With this new experimental design we were able to analyze the active site of a Photoactive Yellow Protein mutant, N43S, during its several stages of the protein functional dynamics. Our research sets a precedent for future experimental groups hoping to utilize rapid-scan FTIR techniques to analyze PYP or other, similar chromophoric proteins.

Contents

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Nomenclature

Physical Constants

h	Planck Constant
$\hbar$	Reduced Planck Constant

Proportionality Constants

k	Spring Constant

Greek Symbols

ν	Frequency
ω	Rotational Frequency
$\widetilde{\nu}$	Wavenumber
γ	Photon

Chapter 1

Introduction

The premise and goal of this research was to create an experimental procedure that would allow future research teams to conduct Rapid Scan FTIR experiments on Photoactive Yellow Protein and its several mutant types. This work was done as a joint project between myself and fellow senior Physics undergraduate Stephen Strecker. The purpose of teaming up was to allow us to focus on two equally vital parts of the experimental procedure, Stephen on the laser and I on the FTIR, because of the time constraints of being an undergraduate and attempting research. Most of the time was setting up the experiment and becoming familiar with the instruments so that this thesis could serve as a guideline for future experimentalists working with PYP and similar proteins. With that being said, much of what is written here is dedicated to the fundamentals of the FTIR and of PYP while my partner's thesis will focus much more heavily on the tunable laser and the triggering. Of course I decided to include a little bit of an overview of the laser's role in this experiment just for a completionist sake, and I urge anyone with further inquiries to go and look at Mr. Strecker's paper.

Chapter 2

Background

2.1 Vibrational Levels and Spectroscopy

We are able to obtain spectra due to the quantum nature of the molecule. Atoms within these molecules are bound to one another by intermolecular forces which do not behave rigidly. These bonds behave in a manner that is very close to a classic mass-spring system which is known physically as a harmonic oscillator. The one dimensional harmonic oscillator has a potential energy

$$V(x) = \frac{1}{2}kx^2 \tag{2.1}$$

However, given the nature of particles at such a small scale, we must make quantum considerations in order to understand the system fully. Particles that may be represented by the system of a quantum harmonic oscillator vibrate at a discrete set of frequencies about the bonds through which they are connected to the other atoms inside the molecule. This discrete set is dictated by a set of real, positive integers, including zero ($n = 0, 1, 2, $), called the quantum number. The vibration that a specific molecule has is directly related to this quantum number and the higher the quantum number, the higher the energy of the vibration. In a quantum system, a one dimensional vibrating particle has the energy

$$E_n = (n + \frac{1}{2})\hbar\omega \qquad n = 0, 1, 2, ... \tag{2.2}$$

Molecules may only vibrate at certain frequencies because of this relationship and because each vibration has a corresponding energy, the energy is only allowed to be at certain values. The vibration corresponding the lowest amount of allowable energy is called the ground state and the one (or ones, as several possible vibrations could have an energy equivalent to the next highest allowable energy) that have the second highest allowable energy are called the first excited

state, and so on to infinity. A molecule is allowed to jump from one energy level to another if it absorbs the amount of energy equal to the difference of the two levels between which the molecule has jumped. An example is that a molecule can jump from the ground state to the first excited state if it absorbs energy from an outside source that is precisely equal

$$E_1 - E_0 = \hbar\omega \tag{2.3}$$

Just as the second highest allowable energy is called the first excited state, the process through which a molecule may change energy levels is called excitation (when it drops down a level, its called relaxation). It just so happens that in most interesting biomolecules this difference of energy in vibrational states is about the energy of a photon in the infrared range (Barth [2007]). We might discern the energy of photon using the Planck-Einstein relation:

$$E_\gamma = h\nu \tag{2.4}$$

A molecule of this description absorbs an infrared-range photon (an acceptable, outside source of energy) whose energy is exactly equivalent to the energy difference between two vibrational levels. In other words, these molecules are excited by infrared light. This phenomenon, called photoexcitation, is not only responsible for our sight, a result of electronic photoexcitation, but is responsible for the entire field of infrared spectroscopy, a result of vibrational excitation. Much how elementary students are taught that the color of an object is responsible for the absorption of all colors other than the one which they are seeing, experimentalists may look at which colors are transmitted (that is, not absorbed) to deduce which ones were absorbed. If we know if a molecule absorbs a photon in a certain range, and are able to calculate the energy of that photon, then we are able to deduce the energy difference of the molecule and thus determine the molecule. Such is the philosophy behind spectroscopy.

2.2 Fourier Transform Infrared Spectroscopy

The merits of spectroscopy as a tool of identification have already been discussed but it is prudent to understand why Fourier Transform Infrared Spectroscopy (henceforth FTIR) is a rapidly growing tool in the science of structural protein dynamics studies. The goal of such studies is to understand the secondary, or three-dimension geometric, structure of proteins. There are several tools in the repertoire of the analyst that allow one to solve for unknown chemical structures among such X-Ray Crystallography, multi-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy, and FTIR spectroscopy (Surewicz et al. [1993]).

However, given the necessity to understand not only the structure of the protein but its place in a dynamic environment gives rise to the need of an equally dynamic tool. The one in the aforementioned set that stands out the most given this constraint is FTIR. Crystallographic studies do not very well mimic the natural environment necessary and NMR studies of larger proteins are often too complex to be feasible (Surewicz et al. [1993]). FTIR allows for the study of proteins in a variety of media and too offers a relatively large amount of information given a relatively small amount of sample (Surewicz et al. [1993]). From spectra gained

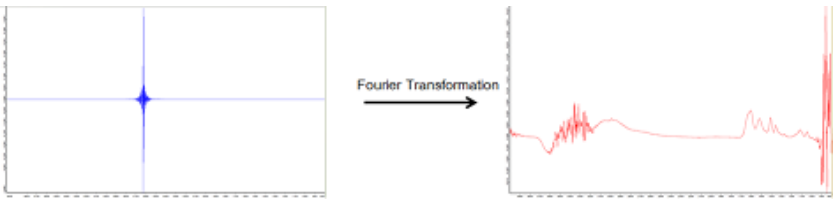

Figure 2.1: Example of an interferogram transformed into a spectrum. This one was taken in our lab suing an AquaSpec sample holder. The sample shown is High pH Threonine.

from the FTIR we are able to investigate the structure of the protein, the molecular mechanism through which proteins react, and also the folding, unfolding, and misfolding of proteins. Through these three studies, we are able to paint a decent picture of what a protein might look like and also how it behaves in various media or in reaction to various stimuli. These spectra are obtained by the vibrational, quantum nature of proteins that are not exempt from the behavior of molecules as stated in the previous section. Those molecules which produce mid-infrared range spectra vibrate at frequencies of about 10^{13} to 10^{14} Hz which correspond to photon wavelengths of about 2.5 to 50 μm (Barth [2007]). The merits of FTIR grow as one investigates the wide range of information that may be determined from the infrared spectrum gathered by the FTIR instrument. The instrument itself is a much akin to a traditional spectrometer whose output signal is Fourier transformed into the spectrum used to analyze the results of the experiment. At its heart is a Michelson interferometer, which is composed of a laser source split into two, orthogonal lasers by a beamsplitter. One of these lasers is redirected to a moving mirror while another is redirected towards a stationary mirror. These mirrors reflect the the lasers back to the beamsplitter where they recombine and are reflected to the sample. The incident beam before the split and the resulting beam after the split differ based on what is called the optical path distance (OPD). The optical path difference is defined by the difference in the distance that the two split lasers travel en route and from the beamsplitter to their re-

spective mirror. The movable mirror may change position by a distance of d and thus the half-beam incident on the moving mirror will travel that extra distance d as well as that distance a second time when it is reflected. When the beams recombine they may or may not be out of phase, resulting in a destructive or constructive interference. The measurement of the intensity relative to the position of the moving mirror is called the interferogram and is the Fourier transform of the spectrum (Barth). Performing a Fourier transform on the interferogram reveals the spectrum for us. A sample may be placed in between the Michelson interferometer and the detector in order to change the composition of the spectrum which is the basis behind all experiments using FTIRs.

Once this light reaches the detector, it produces a spectrum based upon the absorbance that follows the Beer-Lambert Law:

$$A(\widetilde{\nu}) = -log(\frac{I}{I_0}) \tag{2.5}$$

where I is intensity of a specific wavenumber after the sample is inserted and I_0 is the intensity beforehand. Fig. 2.1 above shows such a spectrum on the left hand side. For any "beam" that inserts a Michelson interferometer, the intensity may be represented by

$$I(\delta) = \int_0^\infty B(\lambda)cos(2\pi\lambda\delta)d\delta \tag{2.6}$$

where $B(\lambda)$ is the spectral intensity of the sourcel light.

The purpose of the FTIR is to perform a Fourier Transform on this spectra and the result is may be seen on the right-hand side of Fig. 2.1. The purpose is to find $B(\lambda)$ (Kaledhonkar [2013])

$$B(\lambda) = \int_0^\infty I(\delta)cos(2\pi\lambda\delta)d\delta \tag{2.7}$$

2.2.1 Time-Resolved FTIR

Given the timeframe in which most photochromic systems operate, the FTIR has become a great candidate for any study of photochromic dynamics (Tamai and Miyasaka [2000]). As it will be seen later, the intermediate stages of some chromophores within proteins have lifetimes of anywhere from several pico/nanoseconds to a few seconds. Luckily, the development of FTIRs have allowed for mirror scanning speeds to exceed these ranges and perform full scans in the millisecond range. These modern FTIRs are able to make several complete spectra within a very small time-frame. When these rapid scans are taken sequentially, several spectra

may be made back-to-back, over a time frame that matches the lifetime of an intermediate stage of a protein. With this time-resolved analysis, we are able to make a comprehensive timeline of how biomolecules change how they absorb different wavelengths of IR photons over time. Seeing how these proteins and other biomolecules change the way they absorb or transmit IR light gives insight as to how the structure is changing over time. Such is why structural dynamics of proteins is one of the primary focuses of FTIR laboratories.

As it will be discussed, what is a particular interest of our lab is the chromophore-protein Photoactive Yellow Protein. When this protein receives a photon of a certain energy, it undergoes a process that changes as a function of time. By inserting this extra variable, a single-beam scan is no longer a reliable representation of how the protein is behaving. By using a Time-Resolved FTIR scan, we are able to make several short-term scans. Through increments of scan-times, we are able to therefore determine how the protein is changing over time.

As with all spectroscopy, most spectra require a "background" in order for the data to be accurate. What this means is that the atmospheric conditions must be removed from a spectrum so that it accurately represents what is being looked for. For example, if a protein was to be submerged in water and then measured, one would need to subtract the water spectrum so that it did not mix in with the protein's spectrum and convulute the data. This provides what is called a "dark state", or the conditions to which compare the sample to. In time-resolved measurements, this usually denotes the ground-state conditions before the sample becomes active. When background spectra (or dark state spectra) are subtracted, they leave negative peaks which allow for us to determine that there was something to give a peak before but it has since been removed. Negative peaks, in this sense, can be just as important as positive peaks.

2.3 Photoactive Yellow Protein

Photoactive Yellow Protein (PYP) is a unique protein that was discovered by Terry Meyer in *Ectothiorhodopsira halophila* (E. halophila) which has since been renamed to *Halorhodospira halophila* (Hhal) Hellingwerf et al. [2003]. PYP is a chromophoric protein which means that it contains a molecule called a chromophore which absorbs visible light. Out of all such chromophoric proteins, PYP is the first discovered which possesses the chromophore known as *p*-Coumaric Acid (*p*-CA). When a chromophore absorbs light, it subsequently goes through a processes called photoisomerization in which the protein undergoes a cyclical change in shape. Proteins perform functions by undergoing this process.

PYP is naturally produced in the aforementioned Hhal protein in some small quantities which makes it difficult to extract. However, through the modern use of

gene cloning, we are able to force the overproduction of this protein have allowed for the research of this protein to become rather standard. This, combined with the model nature of its chromophore, have allowed for PYP to become the modern standard for photoactive protein research.

PYP and its chromophore is interesting to study due to the nature of the protein and what it may implicate about the bacteria which house it. Because of the nature of the *p*-CA, and of chromophores in general, one may assume that PYP allows for an organism's response to light. It therefore makes the study of the protein interesting, as further deciphering the protein's photoisomerization might allow for implications of biological behavioral studies to fully be manifested. It need not be said that to attempt to boil down complex behavioral patterns into simple, systematic biophysical responses to physical phenomena or stimuli is an exciting prospect. Indeed some of the first fruits of such labors were published in 2009 (van der Horst et al. [2009]) as it was found that the PYP was resident in a deep see bacteria *Idiomarina loihensis*. This bacteria belongs to a group of γ-proteobacteria and was located at a depth of 1 km in the Hawiaan ocean. As we will see, the PYP absorbs deep-blue light, and so to consider that the PYP allows for triggering of sunlight filtering through the depths of the ocean is an interesting prospect and really produces an exciting field that warrants further study.

2.3.1 Structure of PYP

Photoactive Yellow Protein has a mass of 14kDa, which is relatively small for a protein, and is made up of 125 amino acids. The structure of the protein allows it to be very water soluble. The stable structure has been determined by X-Ray Crystallography and has been shown to have an α/β-fold, containing a six-stranded antiparallel β-sheets flanked by α-helices. The chromophore, *p*-CA is linked to the only cysteine in the protein (Cys69). Hellingwerf et al. [2003]

The composition of the amino acids is: 9 Ala, 9 Val, 7 Leu, 5 Ile, 9 Phe, 1 Cys, 1 Trp, 1 Arg, 4 Pro, 13 Gly, 5 Ser, 7 Thr, 5 Tyr, 6 Asn, 5 Gln, 12 Asp, 7 Glu, 2 Arg, 2 His, 11 Lys. As mentioned, it has 6 β-sheets and 5 α-helices (Kaledhonkar [2013]). It also contains a 3_{10}-helix and one π-helix.

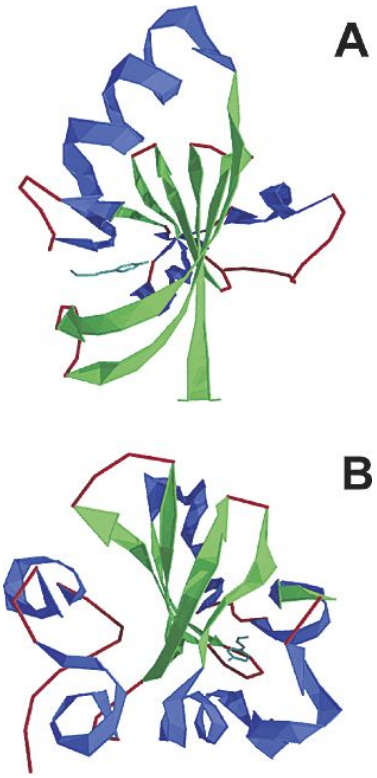

Figure 2.2: The tertriary structure (that means, three dimensional representation) of PYP. One may see the blue α-helicies and the β-strands in green. The chromophore may be seen with some careful inspection, it is the only carbon loop in the structure. Both figure A and B are identical, viewed at different angles.

2.3.2 Photoexcitation and Active Site of PYP

PYP undergoes a reversible photocycle upon absorption of a blue photon. The process through which the active site goes may be divided up into three intermediate stages we may call pR, pB, and pB'. We denote its ground state as pG. This process allows the protein to enter its signalling state and consequently exit it after the reversible process has been completed. pR is the first intermediate stage and it is shown to be a short-lived red-shifted stage, pB a long-lived blue-shifted stage, and then finally the its return to pG. (Xie et al. [1996])

It was not only until 1996 that the photoisomerization of PYP was fully understood. It was at first believed that the pG model could be understood to be $7-trans\ 9-s-cis$ and and then that it would then photoisomerize to a state called $7-cis\ 9-s-cis$. What this means is that in the pG stage, four amino acids were connected by the active site. Glu46, Thr50, and Tyr42 can be thought to be on one "side" of the active site, connected by COOH groups (also called the Amide I band) and that Cys69 was on the other "side". The three on one side and the one on the other were connected in the pG state through a carbon backbone which ultimately was finalized by hyrdrogen bonds connecting CH compounds to Oxygen, which in turn is connected by a carbon backbone to Cys69. It was at first theorized that $7-cis\ 9-s-cis$ was the pR state, in which the Oxygen that was hydrogen bonded to the CH groups was broken and that the connection between all three amino acids were severed. The study in 1996, however, found that Glu46 is perterbered, and that the connection between the three remains.

2.3.3 N43S Mutant PYP

N43S is a mutant of Hhal PYP which implies that the 43rd amino acid in the primary structure (that is, a linear listing of all the amino acids in the protein) has been replaced. The N and S refer to the replaced and the replacement, specifically, Asparagine being replaced with Serine. While the two both are in the same family of amino acids, the mutant was found to have stark differences in comparison to wtPYP (wild type, that found naturally in Hhal). (Kumauchi et al. [2010])

It was first found that the pG dark state of N43S is very similar to that of wtPYP. For a closer look, see Fig. 2.4. The Amide I signals at 1624 cm^{-1} and 1635 cm^{-1} respectively are unchanged. This implies that the mutation does not significantly perturb the secondary structure of the protein. Furthermore the amino acid side chains are also largely unaltered. However what is noticed is that the amplitude of the CO stretching of the side chain in Glu46 is reduced in the N43A mutant.

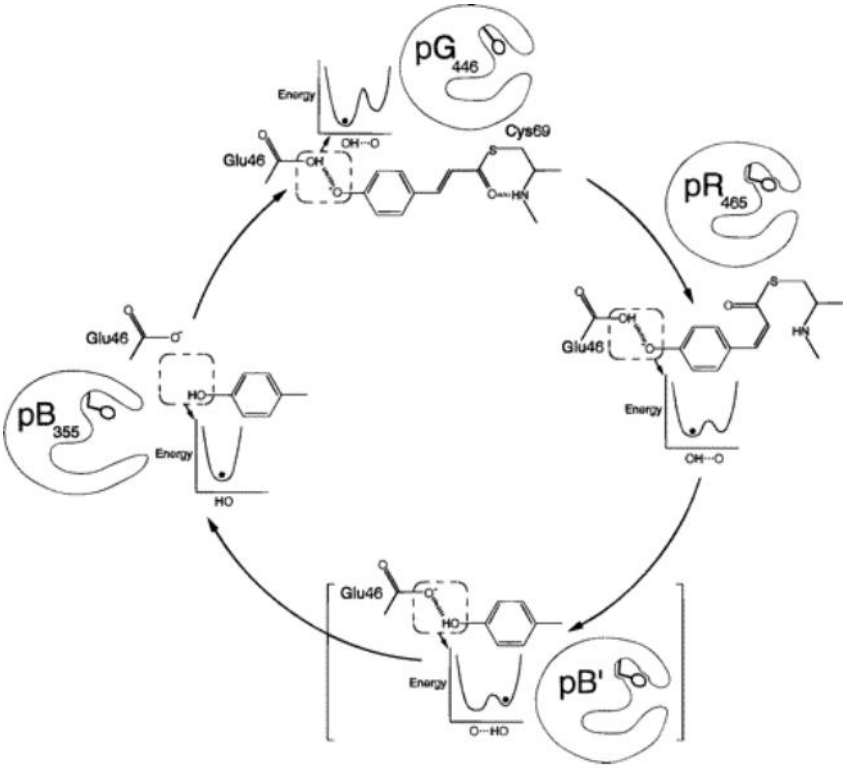

Figure 2.3: As of 1996, this was the predicted PYP functional cycle. The figure describes the energetic potential of the proton being moved through the chromophore as well as a more detailed look at the chemical make up of the PYP. The results were proved later in 2001. Xie et al. [1996]

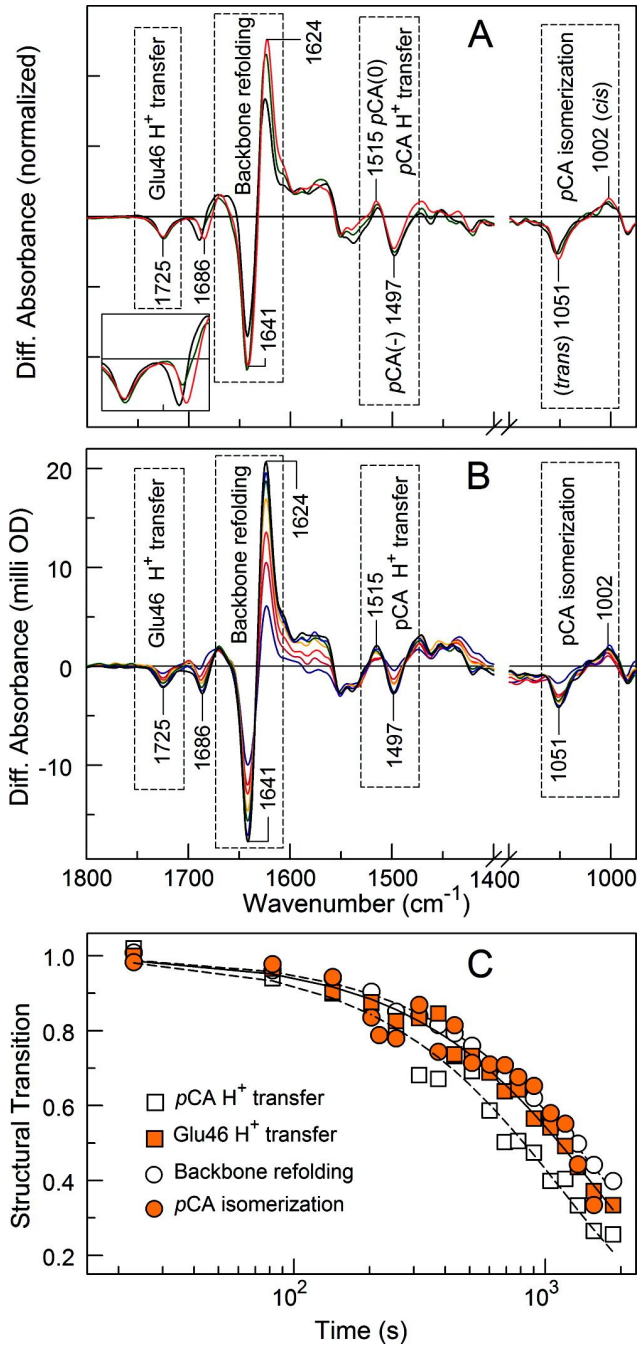

Figure 2.4: The results of the Kumauchi et al. [2010] study, the results that we are attempting to achieve.

Chapter 3

Methodology

Overall the work done by this group is experiemental and the procedure by which it was accomplished is one of the first of it's kind. This brief introduction to the methodology will give a plain overview of the experimental set up of our lab while the later subsections will go into deeper detail.

The foremost thing to mention is that the experimental set up relied on two, vital parts. One is the Fourier-Transform Infrared (FTIR) instrument, and the second is the nanosecond-tunable laser. Through the use of optics we were able to redirect the beam of the laser, set to a frequency of our choosing, into the sample compartment of the FTIR. The PYP, loaded into a sample holder in the compartment to which it lends its namesake, awaits the beam of the laser to excite it into photoisomerization. The FTIR, whose machinations have been explained in the introduction, is then able to get a clear reading on how the PYP changes over the period of its isomerization.

3.1 FTIR

The FTIR Instrument is a Bruker Vertex 80v System, perhaps one of the most advanced of its kind, with which we were able to take this data. The source of light in the system is a MIR Globar (that is, a U-Shaped silicon carbide piece) which is air-cooled. MIR, in this case, stands for Mid-Infrared range, which means the wavelength of the light is from 3-8 μm. The detector is a Photovoltaic Mercury Cadmium Telleride (MCT) detector, cooled by liquid nitrogen (N_2), which is seperated from the sample holder by a BaF_2 window. The effective range of this detector is 12,000-850 cm^{-1}. The beamsplitter that is used in the Michelson Interferometer is KBr whose effective spectral range is 8,000-350 cm^{-1}. Because of the automation of the scanning mirror in the Michelson interferometer, the Vertex 80v comes equipped with a HeNe laser which it uses to detect and assess

the movement and position of the scanning mirror. Because of the HeNe laser, we are able to manipulate the position of the scanning mirror to optimize the amount of infrared light being directed to the detector. The interferometer iteself is an actively aligned UltraScan interferometer with a highest possible spectral resolution better than 0.2 cm^{-1}. The linear scanner which controls the scanning mirror is fit upon an air bearing, connected to a compressed air line, which allows quick and exact movement of the scanning mirror of frequencies of up to 320 kHz.

Typically, as with most biology, spectroscopy requires the sample to be fitted between two windows. One might think back to high-school microscopy when the sample had to be fitted between translucent sample windows in order to isolate the sample and fit it into a controlled environment. In infrared spectroscopy, we must use a similar method, but unlike microscopy, we must use windows that are translucent in the infrared range. We use Calcium Flouride windows (CaF_2) whose effective transmission range is 66,000-1,000 cm^{-1} with a refraction index of 1.40. We use CaF_2 mostly because its most common replacement, KBr, is soluble in water, and CaF_2 is also resistant to most acids and bases.

Due to the nature of the instrument, and of the prevalence of artifacts (unwanted peaks that show up in a spectrum) in the atmosphere, the FTIR operates within a vacuum to preserve the integrity of the various tools within the instrument but also to keep artifacts from the spectrum. The compartment in which the source, interferometer, and the detector are sealed off and vacuumed. Due to the nature of the experiment, the sample compartment, which is not closed off like the rest of the instrument, was open to the elements. We had to redirect a laser beam into the sample compartment so instead of attempting to vaccuum the sample compartment we purged with dry Nitrogen (N_2) gas which is not an infrared artifact.

3.1.1 Experimental Properties of Time-Resolved FTIR Rapid Scan

The resolution at which we scanned the PYP is 4 cm$^-$1 at a speed of 200 kHz. We used an optical filter setting (denoted by the title "Filter 4" by OPUS) which does a good job of reducing the amount of water vapor and other atmospheric artifacts that show up in the spectra. This results in a cutoff at 800 cm^{-1} and below. The set aperture that dictates the amount of light that enters the interferometer has a diameter of 3 mm. The phase resolution was set to 16, which means that the system allowed for 1184 phase interferogram points. The OPUS software utilized a Norton-Beer apodization function and the Phase Correction used the Mertz function. The zerofilling factor is 4.

The Vertex 80v comes with its own operational and analysis software called

OPUS. Through it we are able to tune the various aspects of the FTIR as well as manipulate the resulting spectra to a degree. Most of the figures in the conclusion were made thorugh manipulation of spectra using the OPUS software. For rapid-scan, the OPUS allows for a reasonable amount of flexibility. We developed a macro using the built in software which allows for sending and recieving triggers. The specifics of triggering will be explained in a following subsection.

3.2 Laser

The laser used is a Vibrant 335LD Tunable Laser from OPOTEK, Inc. It incorporates a patented type II Optical Parametric Oscillator (OPO), which is a linear oscillator formed by a retro-reflecting prism and a flat output coupler. The lasing is made possible by a Neodymium-doped Yttrium Aluminum Garnet (Nd:YAG) solid-state crystal. The majority of the operation consisted of activation and de-activation of the flash lamp and of the Q-Switch. The flash lamp activates the process through which population inversion is achieved and is what excites the electrons for eventual lasing. The Q-Switch may be activated once population inversion has been achieved and is what allows the electrons to relax and, in effect, allow the electron to lase. This particular laser needs 20 minutes of flash lamp activation to reach population inversion.

3.2.1 Lasing Parameters and Triggering

The Vibrant laser is mounted on an optical table and is redirected through 7 mirrors into the sample compartment, passing through 1 aperture. The average laser energy was 64.8% of the maximum 19.5 mJ energy, which was, for the experiment, on average 5.95 mJ. The wavelength of the laser was set at 475 nm.

Due to the nature of the experiment, it was vital for all systems to be running on the same clock. For this purpose we employed a SRS DG-535 Delay Generator to run as the master clock. It would send triggers to the Laser and the FTIR as to when to lase and when to take measurements. The FTIR would then send triggers to a shutter to open before it measured the sample, allowing the laser to go through, and would send a signal for it to close after it had concluded measurement.

Chapter 4

Results

The goal of this research was to prepare an experimental procedure for which to use rapid-scan measurements on PYP using a mutant sample as a test. We are proud to say that we made excellent measurements and through the analysis of the data, very concise figures have been produced.

Fig. 4.1 shows a two-dimensional figure that is very much akin to one shown in the Background chapter. With this experimental set-up, we were not only able to reproduce the results achieved in Kumauchi et al. [2010] but, perhaps, make the data even cleaner. These results were achieved by normalizing the resultant spectra achieved from the N43S sample and by making baseline corrections to they system. The results show wavenumbers ranging from 900-1850 cm^{-1}. The absorption peaks, both positive and negative, are shown. The results that Kumauchi et al. [2010] achieved are very similar but shown in higher resolution here. The peak at 1641 cm^{-1} has been shifted to 1643 cm^{-1} which correlates to the backbone folding of the active site. The peak at 1724.82 cm^{-1} represents the proton transfer from Glu46. The negative peak at 1497.97 cm^{-1} denotes the proton transfer going through pCA, as well as the act of isomerization seen at 1052.98 cm^{-1}.

Fig 4.2 shows a three dimensional figure of the same results but shows the changes over time. The first figure begins at 196 ms and each scan after that takes 98 ms. The software used creates a smooth curve in between what would otherwise be spectra stacked on top of each other; similar to several Fig. 4.1 stacked on top of one another.

Seeing as this begins at 196 ms, in the pB range, isomerization can be seen occuring and eventually finishing to return the active site back to the pG state. From the graph given, we may conclude that the isomerization for this particular protein takes roughly 20 to 30 seconds. The largest peak occurs, obviously, at the backbone folding of the active site which gives the highest infrared resonance of the entire active site of N34S.

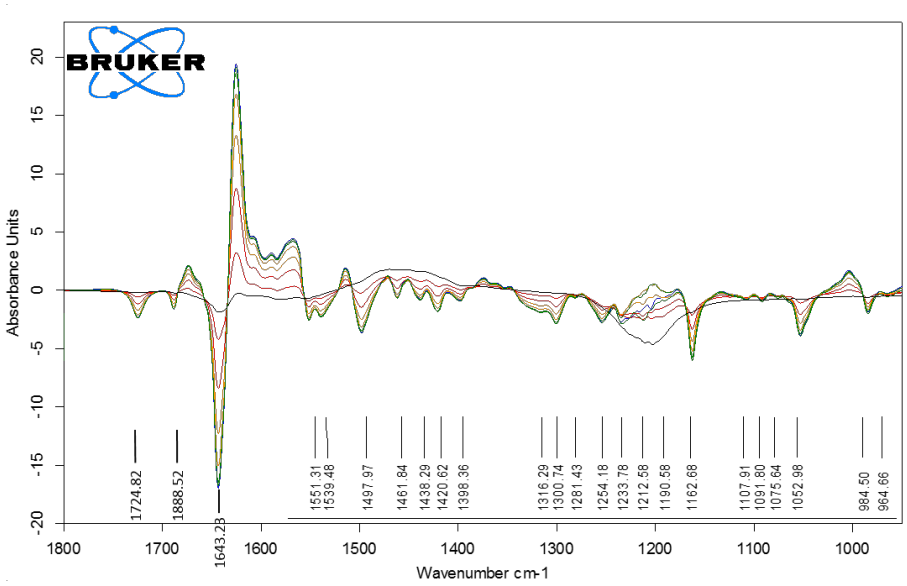

Figure 4.1: A two dimesional model of our absorption spectra for N43S PYP. Compare to Fig. 2.4.

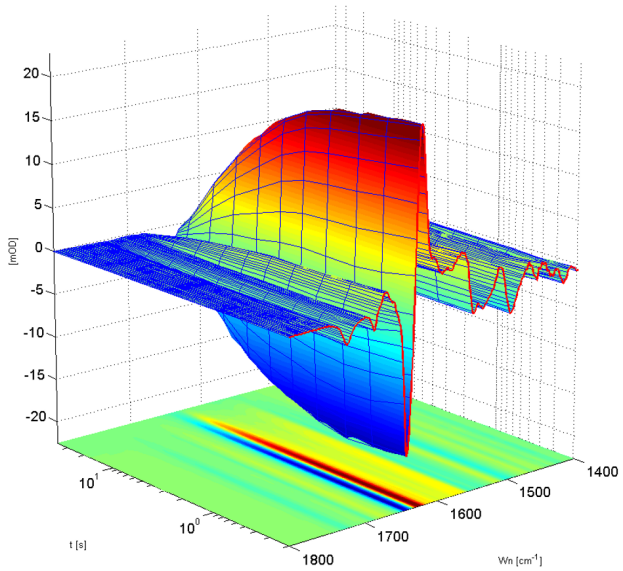

Figure 4.2: A more conventional figure, this 3D graph shows absorbance vs wavenumber over time.

References

A. Barth. Infrared spectroscopy of proteins. *Biochimica Et Biophysica Acta-Bioenergetics*, 1767(9):1073–1101, 2007. ISSN 0005-2728. doi: 10.1016/j.bbabio.2007.06.004. URL <GotoISI>://WOS:000249671400001. 3, 4

K. J. Hellingwerf, J. Hendriks, and T. Gensch. Photoactive yellow protein, a new type of photoreceptor protein: Will this "yellow lab" bring us where we want to go? *Journal of Physical Chemistry A*, 107(8):1082–1094, 2003. ISSN 1089-5639. doi: 10.1021/jp027005y. URL <GotoISI>://WOS:000181145900002. 6, 7

Sandip Kaledhonkar. *Structural Dynamics of Photoactive Yellow Protien*. Thesis, 2013. 5, 7

M. Kumauchi, S. Kaledhonkar, A. F. Philip, J. Wycoff, M. Hara, Y. X. Li, A. H. Xie, and W. D. Hoff. A conserved helical capping hydrogen bond in pas domains controls signaling kinetics in the superfamily prototype photoactive yellow protein. *Journal of the American Chemical Society*, 132(44):15820–15830, 2010. ISSN 0002-7863. doi: 10.1021/ja107716r. URL <GotoISI>://WOS:000283955600068. iv, 9, 11, 15

W. K. Surewicz, H. H. Mantsch, and D. Chapman. Determination of protein secondary structure by fourier-transform infrared-spectroscopy - a critical-assessment. *Biochemistry*, 32(2):389–394, 1993. ISSN 0006-2960. doi: 10.1021/bi00053a001. URL <GotoISI>://WOS:A1993KH38600001. 3, 4

N. Tamai and H. Miyasaka. Ultrafast dynamics of photochromic systems. *Chemical Reviews*, 100(5):1875–1890, 2000. ISSN 0009-2665. doi: 10.1021/cr9800816. URL <GotoISI>://WOS:000087009200010. 5

M. A. van der Horst, T. P. Stalcup, S. Kaledhonkar, M. Kumauchi, M. Hara, A. Xie, K. J. Hellingwerf, and W. D. Hoff. Locked chromophore analogs reveal that photoactive yellow protein regulates biofilm formation in the deep sea

bacterium idiomarina loihiensis. *Journal of the American Chemical Society*, 131(47):17443–17451, 2009. ISSN 0002-7863. doi: 10.1021/ja9057103. URL <GotoISI>://WOS:000272207300072. 7

A. H. Xie, W. D. Hoff, A. R. Kroon, and K. J. Hellingwerf. Glu46 donates a proton to the 4-hydroxycinnamate anion chromophore during the photocycle of photoactive yellow protein. *Biochemistry*, 35(47):14671–14678, 1996. ISSN 0006-2960. doi: 10.1021/bi9623035. URL <GotoISI>://WOS:A1996VV23700001. iv, 9, 10