Single Molecule Fluorescence Spectroscopy of ClpXP-mediated Substrate Degradation

by

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B.S. Mechanical and Aerospace Engineering
Seoul National University (2007)

Submitted to the Department of Mechanical Engineering
in partial fulfillment of the requirements for the degree of

Master of Science in Mechanical Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2009

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Abstract

Energy-dependent proteases, such as ClpXP, are responsible for the regulated destruction of proteins in prokaryotes and organelles of eukaryotes. AAA+ ATPases in these proteases recognize protein substrates and power their mechanical denaturation and subsequent translocation into a sequestered degradation chamber where polypeptide cleavage occurs. Here, we present the single molecule fluorescence assay for probing the interaction between the ClpXP enzyme and its substrates. A covalently crosslinked ClpX hexamer maintain functionally stable form at the low concentration of single molecule level. Surface passivation through polyethylene glycol (PEG) remove unwanted nonspecific binding of substrates, providng specific immobilization of ClpXP protease on the glass surface illuminated by total internal reflection fluorescence (TIRF). Cy3-labeled engineered substrates containing nondegradable GFP in the prescence of ATPγS form stable pre-engaged substrate-ClpXP complexes where the whole substrate degradation pathway, from unfolding to egress of degraded products, can be monitored without competing with dissociation or additional background characteristic of free labeled substrate in solution. We directly observe some terminal processes that are encountered by ClpXP at the end of substrate degradation process. It is also shown that GFP tail domain stably bind to the ClpX in the presence of ATPγS and even in the absence of ATP hydrolysis. With the developement of single molecule assay for AAA+ protease, we can expand our knowledge on the mechanism of this crucial motor protein family.

Thesis Supervisor: Matthew J. Lang
Title: Associate Professor of Mechanical Engineering, and Biological Engineering

Acknowledgments

I really would like to thank my advisor, Matthew Lang, for providing me the opportunity of joining his lab. I still vividly remember the moment when I visited Matt's office at the first time, and an intersting story about the biophysics he told me. Matt welcomed me who even had no idea on what kinesin was. With his kind guidance and advices, I have learned how to 'research'.

This work would have not been completed without the contribution of my collaborators in the Biology department. I want to thank Robert Sauer, Tania Baker who gave me great suggestions and help for analysis of experimental data. I also want to thank Andreas Martin and Joseph Davis who made many proteins that I played for this thesis.

I was forutunate enough to work with talented and supportive lab mates, David Appleyard, Carlos Castro, Ahmad (Mo) Khalil, Hyungsuk Lee, Ricardo G. Rubio, Marie E. Aubin-Tam, Ted Feldman, Sangjin Ryu, Mariya Barch, Benjamin Pelz, Adrien Georges, Ricardo Brau, and Jorge Ferrer. Exchanging idea with them was really enjoyable and all the supports they gave encouraged me to move on.

Finally, I would like to thank to my parents, sister and Mihee. During 2 years of stay in US, I have realized how deep the love my family have given me. I will probably never be able to repay you, but thank you for all your support and love. When I had hard time in graduate life, Mihee has been an amazing source of emotional support. Without you, I can't stand here now. Thank you for keeping me happy.

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Chapter 1

Introduction

1.1 Background and Motivation

Every living things are composed of cells that have wide ranges of molecular machines. For example, RNA polymerase reads inherited genetic informations on DNA, and synthesizes mRNA that will be eventually used to produce proteins essential to maintain proper cellular environment. Kinesin and dynein are involved in the transport processes along cytoskeletal network and also in the re-arragement of spindle assembly in cell division. There are also proteins called proteases that degrade misfolded or useless proteins.[36] All these molecular machines described above are motor proteins which convert chemical energy stored in ATP into mechanical work. Watching conformational changes of these motor proteins have been a long term goal of many scientists. Recently, advances in detection devices and developement of bright fluorescent probe have enabled the realization of this dream. Using single molecule techniques that have sub-nm resolution, individual enzymatic turnovers of molecular motors are directly observed.[1],[2] However, most of the ATP dependent studies of motor proteins at single molecule level so far has been confined to simple transport processes, where less work is required to overcome transport barriers compared to those for the particularly energetic tasks of degradation machinery such as ClpXP protease in prokaryotes. In this thesis, the first developement and application of single molecule fluorescence assay for ClpXP is presented to probe the kinetics

of ClpXP-mediated substrate degradation. This study can set the stage for more detailed single-molecule studies of ClpXP function and its conformational dynamics.

1.2 ClpXP Protease

AAA+ proteins

The AAA+ (ATPase associated with various cellular activities) family is a large and functionally diverse group of enzymes that are able to induce conformational changes in a wide range of substrate proteins using energy from ATP hydrolysis.[23] As indicated in the Fig. 1-1, AAA+ proteins are involved in various cellular processes ranging from protein degradation and DNA replication to membrane fusion and the movement of microtubule motors.[44],[20] Basically, all AAA+ proteins operate by promoting remodelling in target proteins. In many cases, this remodelling perturbs protein structure sufficiently enough to promote unfolding. For example, the 26S proteasome and many prokaryotic proteases such as ClpXP contain AAA+ ATPase domain to drive unfolding of target substrate and its translocation into associated peptidases. Protein aggregates generated by environmental stress or a mutant protein are also disassembled by the activity of AAA+ protein such as Hsp104 or ClpB. The exact mechanism of how these AAA+ enzyme mediate the dissociation of protein aggregation has yet to be elucidated, but a similarity between AAA+ mediated disaggregation and the unfolding reactions has recently been found.[54] Thus, the advances in our understanding on how AAA+ ATPase exerts mechanical force on its substrate by converting chemical energy of ATP will provide us a more complete description of various cellular processes.

Structure of ClpXP proteases

AAA+ proteases play critical roles in maintaining the proteome in all organisms.[48] They degrade short-lived regulatory proteins and thereby control cellular processes such as signal transduction, cell cycle, and gene transcription. The proteases also remove misfolded and aggregated proteins from the cell and produce some of the

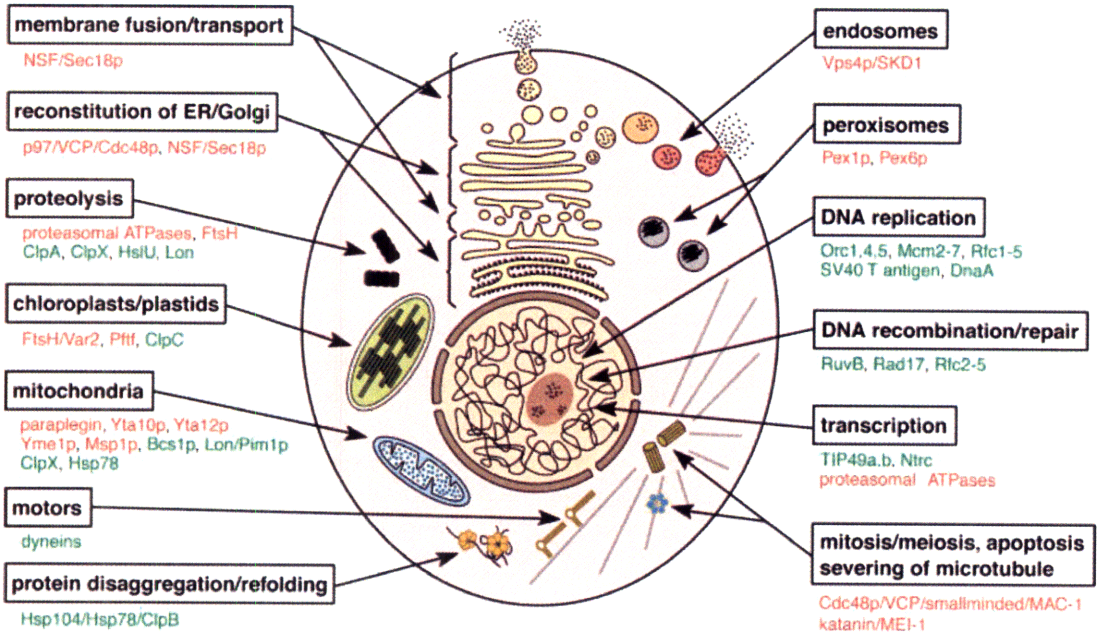

Figure 1-1: The diverse cellular functions of AAA+ proteins. The subcellular localization of AAA+ proteins and the processes in which they participate are indicated. AAA members are in red and other AAA+ members in green.(Figure adapted from [44])

peptides to be presented at cell surface as part of adaptive immune response. In eukaryotes, these functions are fulfilled mainly by the proteasomes, and in prokaryotes and the organelles of eukaryotes, the functions are carried out by analogs of the proteasome, such as the ClpXP, ClpAP, HslUV, FtsH, and Lon proteases. ATP-dependent proteases share a common architecture although they show only relatively little sequence identity.[25] The active sites that catalyze peptide bond cleavage are sequestered in a hollow interior chamber, typically constructed from rings of six or seven subunits.(Fig. 1-2) Substrates enter these degradation compartments through axial channels that are too narrow to admit folded native proteins. This restriction prevents the undesirable destruction of most cellular proteins but requires the coordinated enzymatic recognition, denaturation and translocation of correct substrates before degradation. Hexameric rings of subunits or domains, which belong to the AAA+ ATPase family, perform these mechanical unfolding and translocation

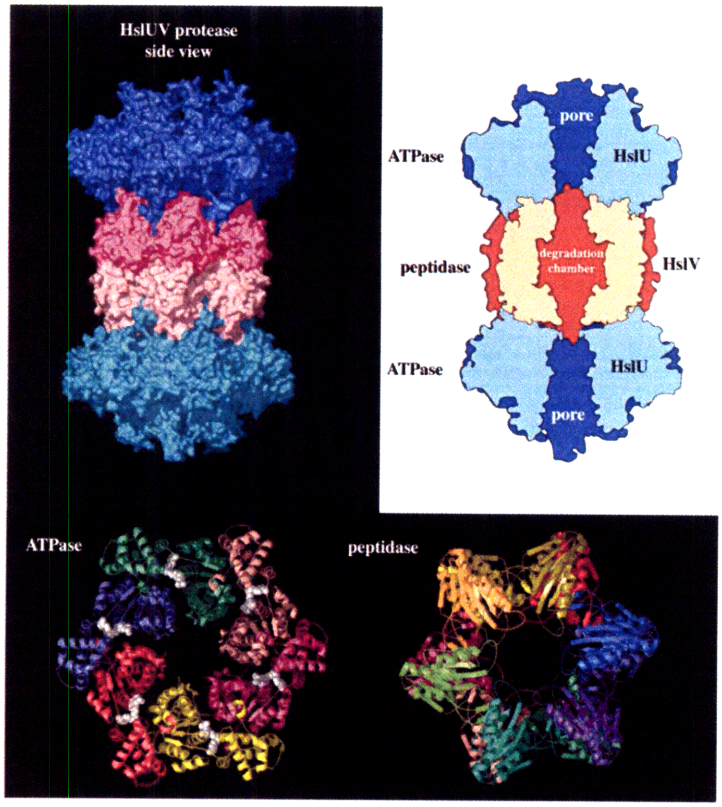

Figure 1-2: Structural views of the HslUV protease and its components. The top left panel shows a surface representation with individual hexameric rings of the HslU ATPase colored blue/cyan and the HslV peptidase colored magenta/pink. The top right panel is a cutaway diagram showing the positions of the pore through HslU and the degradation chamber within HslV. The bottom panel shows axial views of the ATPase and peptidase in ribbon representation with individual subunits in different colors. (Figure adapted from [48])

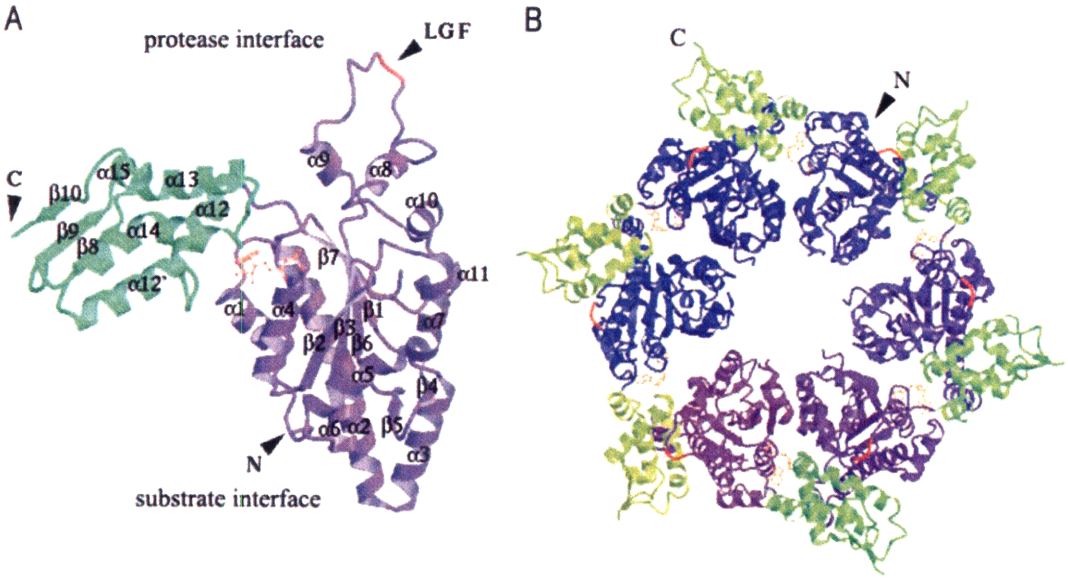

Figure 1-3: The structure of ClpX AAA+ ATPase. The ribbon diagram of the ClpX monomer(A) is presented. The ATPase core domain, SSD domain, and IGF tripeptide are colored magenta, green, and red, respectively. The ATP molecule is shown in orange as a ball-and-stick model. The ribbon diagram of the hexameric construction of ClpX (B) viewed from the protease interface are shown. The same color schemes are used. (Figure adapted from [32])

steps.[4]

Like other proteins in the AAA+ familiy, each subunit of ClpX is composed of an ATPase core domain in the N terminus and a SSD(sensor and substrate discrimination) domain of the C terminus (Fig. 1-3).[32] There is an additional small Cys cluster domain at the N terminus, but it is considered that this Cys cluster domain is not necessary for the proteolytic activity of ClpXP protease, or for hexameric assembly.[49] This two sub-domain architecture of ClpX is highly conserved in most of AAA+ proteins. Wedge-shaped N terminus domain that has β-sheet of parallel strands is also the distinct structural feature of AAA+ enzymes.[23] The main differences between ClpX and other AAA+ proteases are the presence of a ClpP-binding loop in the ClpX and the β sheet structure at the C terminus. A functional ClpX protein is actually a hexamer of 6 ClpX monomers whose oligomerization is facilitated by nucleotide binding. This 140$\AA$-wide-70$\AA$-thick enzyme has a central pore where lining amino acids are believed to be engaged in the recognition, denaturation and

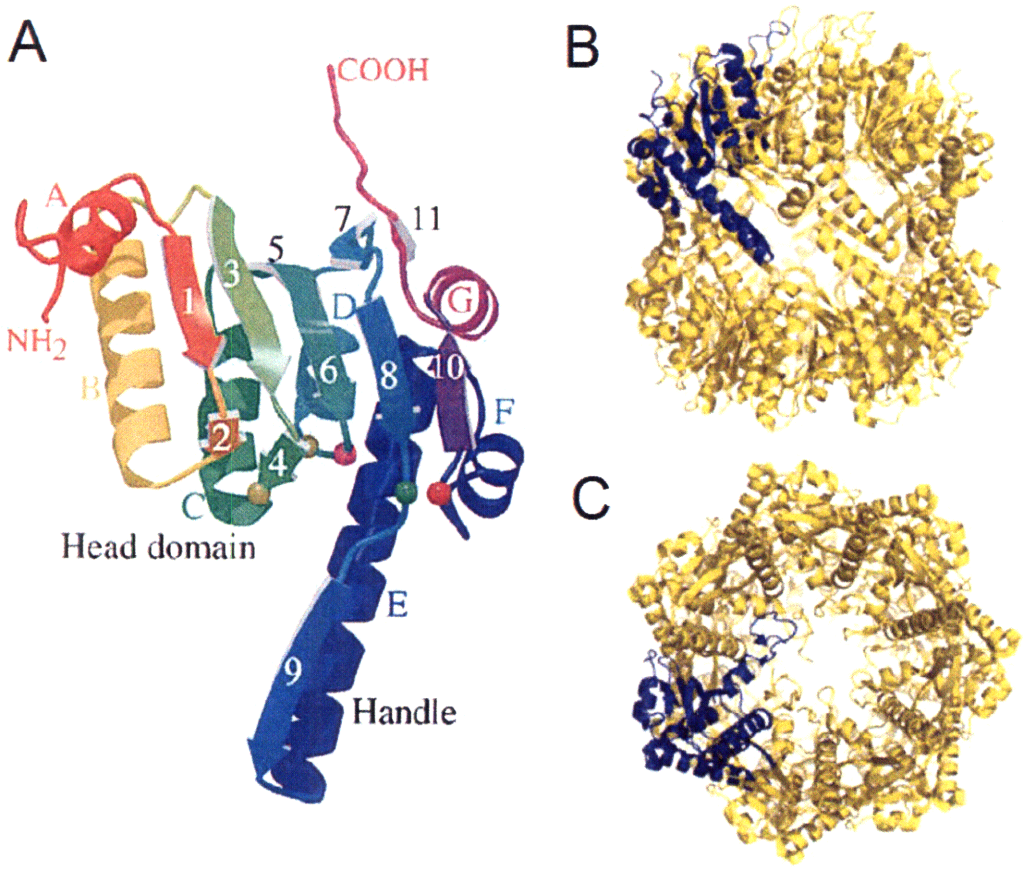

Figure 1-4: The structure of tetradecameric ClpP peptidase. (A) The ribbon diagram of the ClpP subunit shows its secondary structure. The helices and strands are shown from N to C terminus with rainbow coloring. In addition, residues in the catalytic triad (Ser 97, magenta; His 122, green; and Asp 171, red) are drawn as spheres. Side (B) and top (C) view of the X-ray structure of assembled ClpP from E. Coli are shown. Single monomer is highlighted in blue. (Figure adapted from [53],[55])

translocation of substrates. It is recently shown that the tyrosine residue of a conserved aromatic-hydrophobic motif in the pore physically gribs substrates and thereby transmits mechanical force to polypeptides during unfolding and translocation.[39]

The ClpP belongs to the serine protease family; in the E. Coli enzyme, the catalytic triad consists of Ser 97, His 122, and Asp 171.[41]. This peptidase subunits assemble in vivo into two seven-membered rings.(Fig. 1-4)[53] The internal chamber of the assembled ClpP, lined with the 14 proteolytic active sites, is sufficient to accommodate about 51kDa of protein, but the entry portal of free ClpP has a diameter

of only 10$\mathring{A}$, large enough to allow passage of only a single polypeptide. Interestingly, several chains can be translocated concurrently during ClpXP degradation of proteins containing disulfide bonds.[9] Hence, the ATPase must increase the size of the peptidase pore in some fashion. Peptide bond cleavages in active site of ClpP does not show strict sequence specificity, although substrates are cleaved preferentially after non-polar residues.[51] Docking of the hexameric ClpX and heptameric ClpP involves a symmetry mismatch. Flexible loops containing the tripeptide IGF make hydrophobic interactions with hydrophobic clefts of the ClpP asymmetrically. These loops help align the pore of ClpX with that of ClpP, and also mediate functional communication between unfoldase and peptidase during the substrate processing cycle.[27]

Substrate recognition

Since the ClpXP protease is a cytosolic enzyme, it is critical to regulate the engagement of the proper substrates for degradation. This control occurs at the substrate recognition level. Since bacteria have no ubiquitin system, recognition of specific protein targets for destruction is mediated by a diverse set of unstructured peptide signals displayed at the N-terminal or C-terminal end of a target substrate. The importance of short peptide sequences in bacterial protein destruction is exemplified by the SsrA quality control system, which adds a degradation tag to nascent polypeptides on stalled ribosomes.[28] When recruited to a distressed ribosome, SsrA acts as tmRNA to direct addition of the tag sequence AANDENYALAA to the C terminus of the nascent protein. This ssrA tag, in turn, targets the attached protein for degradation by ClpXP, ClpAP and other proteases. No additional substrate information is necessary for degradation, and thus the ssrA tag functions as a strong primary degradation signal.[48] However, ssrA tag is not the only one that is recognized by ClpXP protease. Indeed, recent proteomic study revealed five different classes of naturally encoded peptide motifs which direct degradation by ClpXP.[18] This result can be rationalized from a biological perspective, as the existence of multiple classes of degradation signals would facilitate differential regulation of the degradation of disparate classes of substrates depending upon the demands of cellular environment.

Thus, one role of ClpXP may be to readjust the composition of the proteome following the global changes in gene expression that accompany responses to stress.

Substrate unfolding, translocation and degradation

After substrate recognition by ClpX, native substrates must still be denatured, translocated into the degradation cavity of ClpP and ultimately proteolyzed. This interaction of the ClpXP with its substrate was directly imaged by using electron microscopy.[45] When substrate λO protein was added to the ATPγS-stabilized ClpXP complexes, extra electron density appeared to the distal surface of ClpX. Upon addition of ATP, this density disappeared as λO protein was degraded. Interestingly, when ATP was added to proteolytically inactive ClpXP-λO complexes, the extra density transferred to the center of ClpP and remained inside ClpP even after separation from ClpX. Further studies were done using synthesized chimera protein conjugated to ssrA degradation tag. Since ssrA tag alone can be the sole determinant for being a substrate of ClpXP, any proteins can be used as substrates for ClpXP after appropriate modification on the C-terminus. Green fluorescent protein (GFP) has been widely used as a model substrate since its conformation can be monitored in real time by changes in fluorescence.[33] Using GFP-ssrA substrate, it was shown that ClpX is indeed a protein unfoldase, and substrate denaturation is the slow, rate-limiting step in the overall degradation reaction.(Fig. 1-5) In addition to its role in catalyzing substrate denaturation and translocation, the ClpX ATPase also catalyzed active release of trapped GFP-ssrA from inactive ClpP. It seemed that ClpX ATPase cycle opens the narrow central pores of the ClpP peptidase to allow denatured substrates to enter the degradation chamber and the same mechanism could also permit release of cleaved peptide products from the ClpP.

Several studies support a model for protein unfolding by ClpX in which the global thermodynamic stability of the substrate plays a very little role in resisting denaturation. Instead, it has been suggested that the local stability of substrate structure near the degradation tag plays an important role in resisting denaturation. Once these local folds are unfolded, global denaturation of single-domain proteins occurs

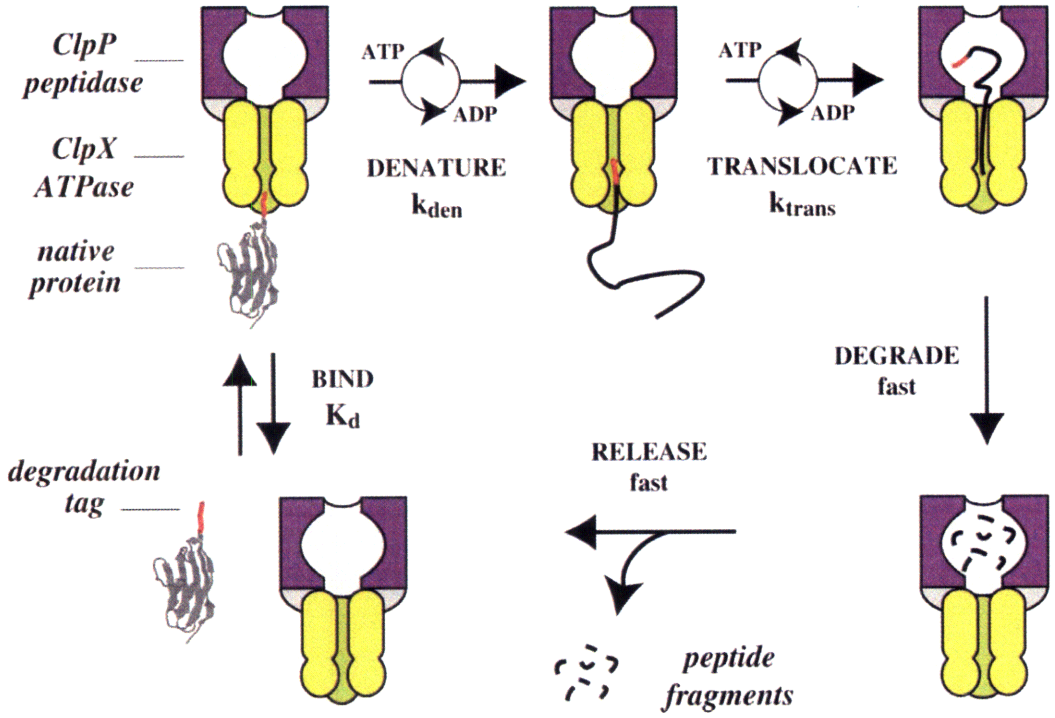

Figure 1-5: A current model for substrate denaturation and proteolysis by ClpXP. The first step in the reaction is substrate recognition and binding. This is followed by the mechanical denaturation of the substrate. Finally, the unfolded polypeptide is translocated through the ClpX pore and into the ClpP degradation chamber. (Figure adapted from [29])

rapidly thereafter in a spontaneous reaction because of the cooperativity of protein unfolding.[29] There has been some debate about the mechanism on how each ClpX subunit communicates each other to ultimately degrade substrates. Even though there are six identical subunits in a ClpX enzyme, it is becoming clear that AAA+ hexamers function in an asymmetric manner. Recent studies suggest that some of subunits do not bind ATP, some bind ATP tightly and some bind ATP weakly.[24] In addition, hexamers of covalently linked ClpX monomers with active or inactive functionalities have shown that diverse geometric arrangements support substrate degradation. Indeed, it was found that linked ClpX with only a single subunit capable of hydrolyzing nucleotides still has ability to unfold substrates. In fact, the capability of a hexamer to destruct proteins increases with the number of active

subunits in the hexamer, but is independent of the relative locations of the active subunits. Taken together, these results preclude a model of concerted hydrolysis in which all or a fraction of subunits simultaneously hydrolyze ATP. They also disfavor sequential hydrolysis in which hydrolysis in one subunit leads to hydrolysis in an adjacent subunit and so on. It seems that the most likely mechanism is one in which hydrolysis is probabilistic rather than predetermined.[38]

1.3 Single Molecule Fluorescence

Brief history

It has been many scientists' dream to detect, manipulate, and control individual molecules. Since the first successful detection of single fluorophores at cryogenic temperature by Moerner's group in 1989,[42] there has been an explosive growth in single molecule fluorescence studies. A major advance in the single molecule detection came with the imaging of immobilized single fluorophores by near-field [5] and later far-field scanning optical microscopies at room temperature.[37] Wide-field microscopy of single fluorophores with total-internal-reflection (TIR) excitations were demonstrated by improved charge coupled device (CCD) cameras and carefully removing major sources of background. With these techniques, the first biological applications of single molecule fluorescence techniques were demonstrated with surface-bound molecules. Individual ATP turnovers by a single myosin molecule,[19] and the movement of single kinesin molecule along microtubules[52] were observed at video rate. Single molecule fluorescence resonance energy transfer (smFRET), first demonstrated in 1996,[21] has been widely adopted by many research groups to study a variety of biological systems rich in structural dynamic changes. The capability of smFRET, with which nano-meter-order distance changes can be measured in real time, allow researchers to study protein folding,[13] RNA folding,[56] and stepwise subunit rotation of ATP synthase.[15] Detection methods for freely diffusing single molecules in solutions were also further developed. When a fluorophore traverses through a confocal volume, a fluorescence photon-burst is generated. This burst analysis with confocal

fluorescence microscopy has been used to study fluorescence saturation and diffusion of single molecules in solution.[43] Fluorescence correlation spectroscopy (FCS) using similar confocal setup is well suited for the study of molecular mobility, diffusion both in simple artificial systems and directly in living cells.[3]

Basic principle of fluorescence

Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light that is typically of shorter wavelength. The difference between the exciting and emitted wavelengths, known as the Stokes shift, is the critical property that makes fluorescence so powerful. By completely filtering out the excitation light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent. This approach gives such high contrast that even single fluorescent molecules are visible if the background has no autofluorescence. A useful approach to understand the details of excitation and emission process of fluorophores is to render the process in the form of a diagram first conceived by Alexander Jablonski in the 1930s.(Fig. 1-6) On the left side of the diagram are the singlet states. These states maintain the paired +1/2 and -1/2 spin states of the electrons as they are normally, with each electron in a pair having opposite spins. S_0 is the ground state and represents the energy of a molecule that is not being excited by light. S_1 and S_2 are excited singlet states in which an outer electron is boosted into a different orbital. Higher states in the diagram have higher energy. On the right of the diagram are the triplet states in which an outer electron, boosted to a new orbital, has also undergone a subsequent reversal in spin so that a former pair of electrons are now parallel. From quantum theory, this is a 'forbidden' transition which is relatively unlikely. Nonetheless electrons can undergo 'intersystem crossing' between singlet and triplet states.[35]

When a fluorophore absorbs light, all the energy possessed by a photon is transfered to the fluorophore. If the absorbed photon's energy is greater than that needed to exactly transition from the ground state to the lowest energy level of S_1, the molecule will also undergo a change in vibration, rotation and/or move into an even

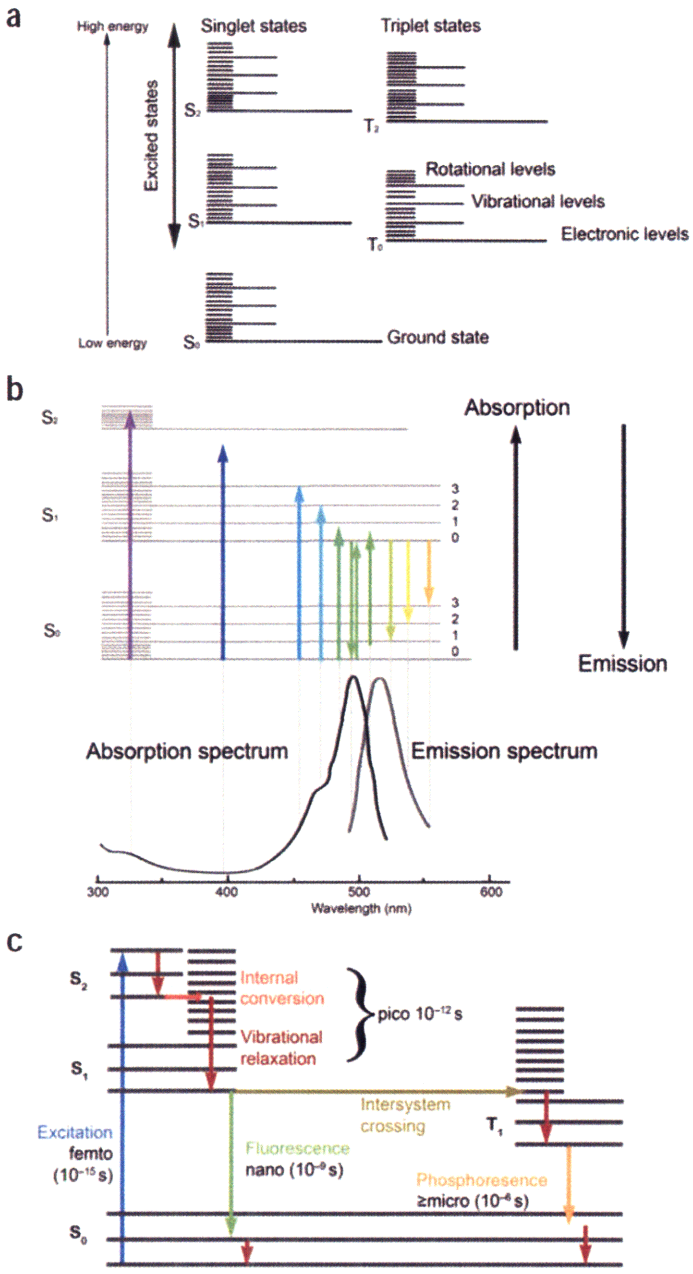

Figure 1-6: Principles of fluorescence. (a) Jablonski diagram displaying the energy states of a molecule. Molecules not absorbing energy are mainly confined to the lowest vibrational states of the ground state S_0. (b) The spectral characteristics related to absorption and emission of energy by a molecule can be related to the size of energy steps needed to bring a molecule from one energy level to another. The absorption and emission spectra of the example fluorophore are shown below a Jablonski diagram. Note the symmetry between the absorption and emission curves owing to the similarity of transitions to vibrational states in S_0 and S_1. (c) The times that the various steps in fluorescence excitation and emission and phosphorescence take. (Figure adapted from [35])

higher electronic orbital (S_2). Thus, there is a range of wavelengths that can excite a molecule. Typically, the energy for excitation has a sharper cutoff on the low energy side of the spectrum than high energy side since there is a threshold energy needed to get excitation whereas there are many possible configurations for high-energy-short-wavelength side of the spectrum. Once excited, the molecule uses several different pathways to ultimately lose the absorbed energy and return to the ground state. Vibrational relaxation allows transition from high vibrational mode of excitated electronic state to the lowest energy level of S_1. This relaxation process takes picoseconds and ultimately the excited molecules drop back to the ground state with the emission of fluorescence light. Due to the energy loss through the vibrational relaxation, the energy of the emitted photon is typically less than the absorbed photon - this is the origin of the Stokes shift.

Fluorescence emission is not the only way fluorophores in the excited state can lose energy. Sometimes, intersystem crossing by means of a forbidden transition to the triplet state occurs. Since molecules in the triplet state are long-lived dark states, they cannot cycle through absorbance and emission rapidly, thus result in unreliable fluorescent probes. However, the most problematic issue of triplet state is related to the permanent loss of fluorescence called photobleaching. Since the long-lived triplet state provides more opportunities for a molecule with an excited electron to interact with other molecules than the briefer singlet states, most photobleaching is thought to be associated with triplet states. It appears that one of the important ingredients in photobleaching is an interaction between a triplet state fluorophore and molecular oxygen. The triplet state can tranfer its energy to oxygen (which is itself a triplet in the ground state), exciting oxygen to its singlet excited state. Singlet oxygen is a reactive molecule than can participate in many kinds of chemical reactions with organic molecules. These chemical reactions can covalently alter the fluorophore to inactivate its ability to fluoresce.

Total internal reflection fluorescence (TIRF)

TIRF techniques have been widely used to obtain statistics with a high S/B (signal-to-

background) ratio for a large number of molecules but with a low-temporal resolution. Typically, this is achieved by exciting fluorophores dissolved in a liquid medium proximal to a surface with a high index of refraction, such as glass. The mismatch in index of refraction can be exploited to create an evanescent wave that decays exponentially by using total internal reflection at the interface between the two media. Molecules in a thin layer of approximately 200nm thickness immediately next to this interface can still be excited by this evanescent wave. Hence, the fluorescent molecules above the thin excitation zone cannot be excited thus providing very good S/B ratios. The movement of fluorescently labeled microtubule-motors[1] as well as individual ATP turnover reactions[19] were probed using TIRF.

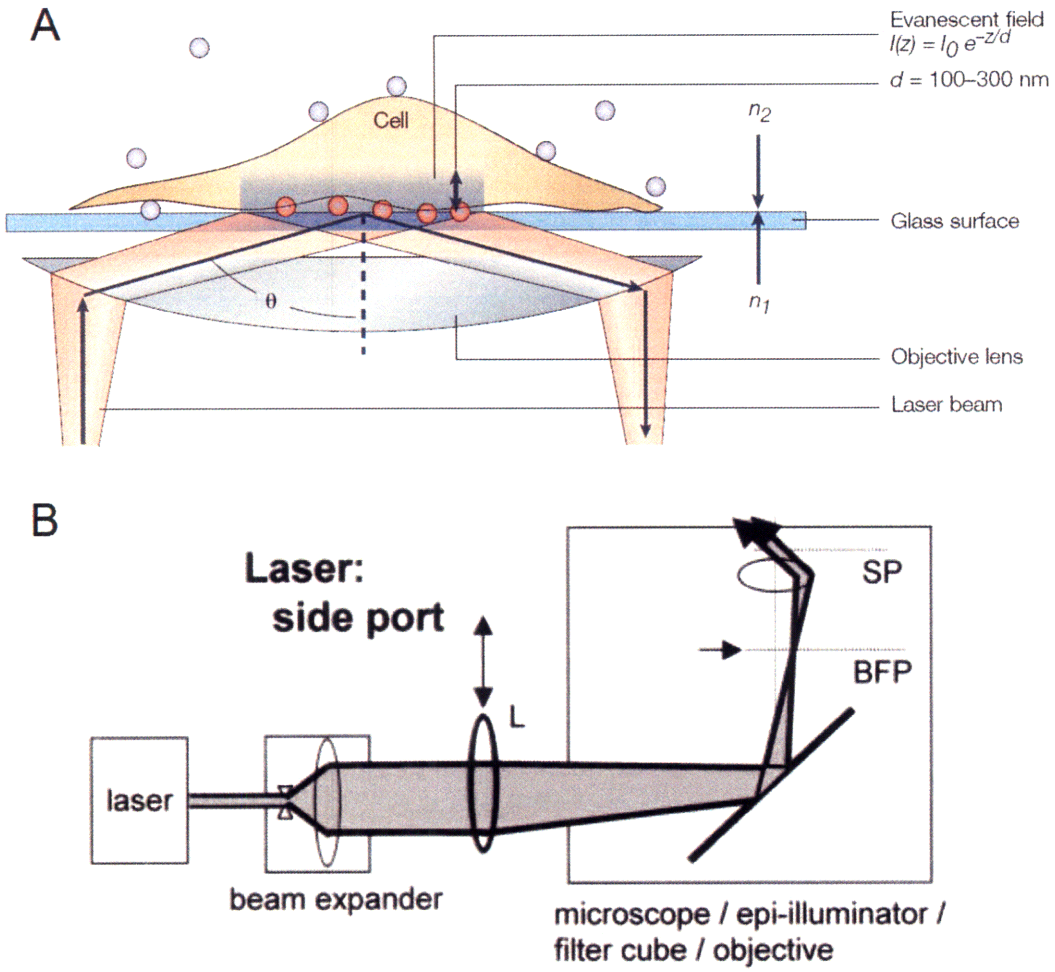

Figure 1-7: Total internal reflection fluorescence microscopy, through-the-objective configuration. (A) When a light beam illuminates the interface of two media obliquely from a high (n_1) to a low (n_2) diffractive index with an incident angle (θ) greater than the critical angle (θ_c) of total internal reflection, then an electromagnetic field called the 'evanescent field' (I) rises from the interface into the medium with a lower diffractive index. This evanescent field decays exponentially with distance (z) from the interface. The decay length (d) of the evanescent field is dependent on θ. In objective-type TIRF, a laser beam illuminates the specimen through the objective lens which is also used in collecting images. As the θ_c of total internal reflection from glass (n_1 = 1.52) to water (n_2 = 1.33) is 61°, an objective lens that has a numerical aperture (NA = $n_1 sin\theta_c$) larger than 1.33 should be used for objective-type TIRF. (B) Typical arrangement for objective-type TIRF based on an inverted microscope. Collimated laser beam is focused at the back focal plane (BFP) of objective through a lens (L) mounted on translator. The position of this focused beam should be sufficiently off-center to reach supercritical angle propagation into the coverslip. Moving the lens L transversely changes the angle of incidence at the sample plane to allow for switching between Epi and TIR illumination. (Figure adapted from [35],[47])

Chapter 2

Experimental Methods

2.1 Enzymes and Substrates

ClpX, ClpP and substrates were expressed and purified as described elsewhere.[40] In order to facilitate the immobilization of ClpX hexamers, BirA acceptor peptide (HAAGGLNDIFEAQKIEWHEDT) was fused into C-terminus and enzymatically biotinylated.[11] Among sites where enzyme activity was not perturbed, the position of biotinylation was decided to make the orientation of central pore of ClpXP enzyme parallel to the glass surface, preventing any steric hindrance that may be generated due to some poor orientations. All engineered substrates contained cysteine residue at N-terminus, and were labeled with a Cy3 maleimide according to the manufacturer's instructions (GE Healthcare).

2.2 Flow Cell and Surface Passivation

An air-tight flow cell was created with a pre-drilled slide and PEG-coated coverslip as follows.(Fig. 2-1) Two holes separated by around 4cm were drilled on a normal microscope slide with a diamond drill bit (1.6mm, UKAM) to allow for the desired buffer exchange. The size of these holes were just large enough to tightly fit with 0.07" tubing (VWR) which would be connected to a syringe. Before the assembly of flow chambers, all pre-drilled glass slides were etched with KOH ethanol solution

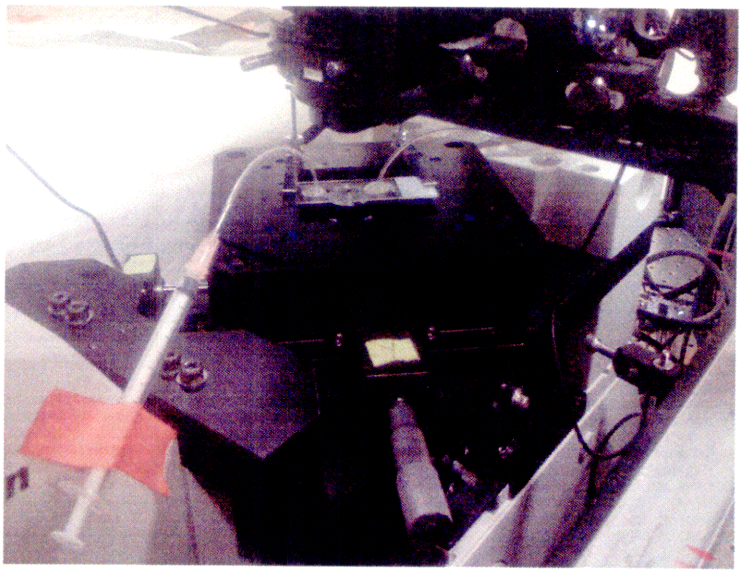

Figure 2-1: A fully assembled flow cell mounted on the sample plane. Glass flow cells assembled with a PEG coated coverslip and a drilled glass slide was used in the preparation of a single molecule imaging sample. Just before adding ATP and Mg^{2+} to trigger ClpX unfolding of GFP, tubes were connected and sealed with epoxy as shown in thig figure. Holding with stage clips and using long tubes prevents any drift which might be generated while pressing a syringe.

and stored in vacuum for the later use. Polyethylene glycol (PEG) coated surface was used to minimize the nonspecific protein absorption.[10] For this PEGylation of coverslips, they were at first etched with KOH ethanol solution to functionalize glass surface and then dipped into 2% aminosilane in acetone solution to obtain amine coated coverslips. Monofunctional mPEG-SVA (Laysan Bio) dissolved in 100mM sodium bicarbonate (pH 8.7) were mixed with biotin-PEG-SVA in same buffer to obtain 1% biotin-PEG mixture (biotin-PEG : mPEG = 1 : 100). The mixed PEG solution was applied to the amine-modified coverslips and incubated for at least 4 hours. A sample chamber was then constructed by sandwiching a pre-drilled glass slide and a PEG-treated coverslip with double sticky tape and by sealing with epoxy. After measurements, used glass slides can be recycled by carefully removing double sticky tape and a coverslip.

2.3 Instrumentation

Objective-type total internal reflection fluorescence microscopy was used to acquire the fluorescence signal of Cy3 conjugated to ClpXP associated substrate.(Fig. 2-2) The 532 nm excitation laser is guided off-axis into a 1.45 NA 100X objective (Nikon) via a dichroic mirror held in a filter cube set (Chroma Technology). In order to minimize photobleaching, the power delivered to the specimen plane was adjusted to 50μW with an acousto-optic deflector (AOD) and also modulated to be pulses with a period of 3.3s for GFP and CFP-GFP degradation, and 9.3s for I27-GFP degradation. Fluorescence emission is collected through the same objective and spectrally filtered with a long-pass filter to block any reflected excitation laser light before being imaged onto a EMCCD camera (Andor Technology). The camera is externally triggered such that it only acquires images of the specimen plane for the duration of the excitation laser pulse, 300 ms. The resulting series of images contained many single molecule spots corresponding to individual Cy3 molecule conjugated to substrates associated with immobilized ClpXP. These were then analyzed to determine the longevity of each fluorescence spot with custom MATLAB code.

2.4 ClpXP Substrate Degradation Assay

Flow cells were prepared as previously mentioned. To obtain the pre-engaged substrate-ClpXP complexes, 1μM substrate was incubated with 0.31 μM ClpX, 1 μM ClpP and 2mM ATPγS for 45 minutes in PD buffer (25mM HEPES, 100mM KCl, 10mM $MgCl_2$, 10% glycerol (v/v), 0.1% Tween (v/v)) at 30 °C as described in [40]. After treating the constructed flow cell with 20μL of 0.01mg/ml streptavidin, the diluted pre-engaged substrate-ClpXP complexes were immobilized on the PEG surface through specific biotin-streptavidin binding by incubating for 20 minutes at room temperature. After washing a flow cell with 50mM EDTA and 2mM ATPγS to chelate Mg^{2+} and stop ATP hydrolysis, the ClpXP-substrate complexes were then washed with PD buffer without Mg^{2+} plus 6mM EDTA and 1mM ATP to exchange

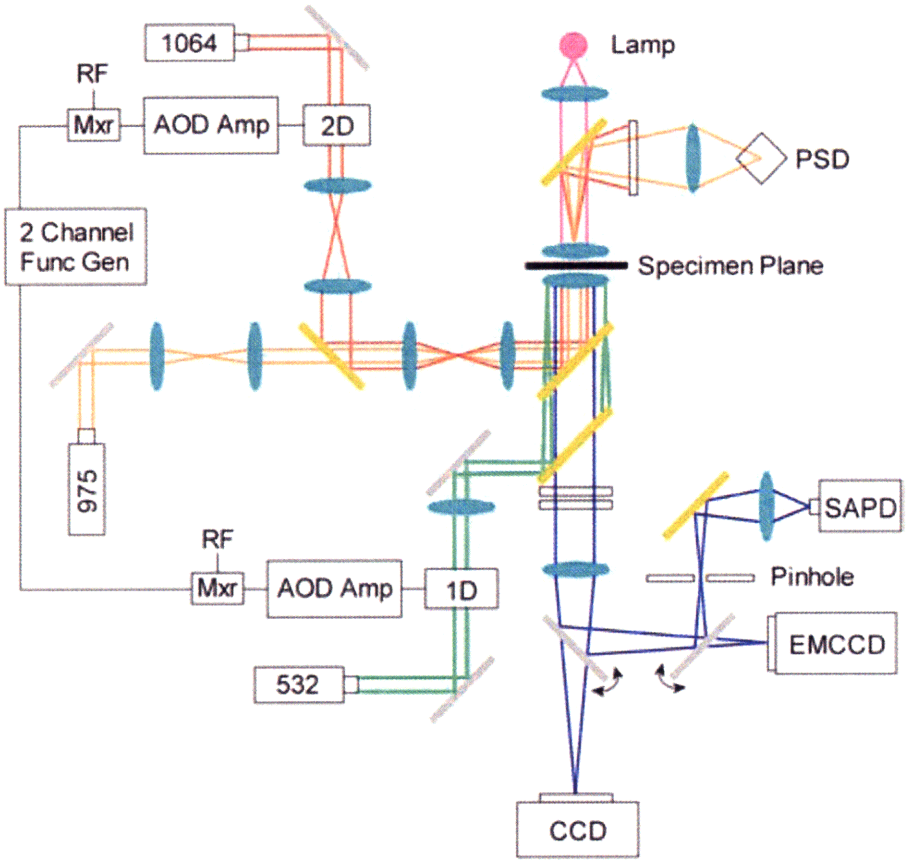

Figure 2-2: Single molecule fluorescence optical layout. All lenses are displayed as blue ovals, and filters, mirrors, and dichroics as white, silver, and gold rectangles, respectively. Total internal fluorescence excitation, supplied by a 532nm laser (green), is focused near the back focal plane of the objectives. Fluorescence images (blue) are acquired by an electron multiplying CCD (EMCCD), and single molecule fluorescence counts are spatially filtered through a pinhole and detected by SAPDs. (Figure adapted from [7])

bound nucleotides from ATPγS to ATP. To keep the ClpXP-substrates complexes from dissociation, we added an extra equimolar amount of ClpP in all these washing buffers. As for ATP, ATP regeneration system containing 2.5 mM creatine phosphate and 0.05 mg/ml creatine kinase was always accompanied. At this point, tubings were connected to the holes of the flow chamber and sealed with epoxy to prevent any leakage. Then, the constructed flow cell assembly was mounted on the microscope and fixed tightly with stage clips to minimize drift during measurement. After identifying the suitable surface region for analysis, ATP hydrolysis was initiated by flowing in 100μL of PD containing Mg^{2+} and appropriate amount of ATP.(Fig. 2-3) An oxygen scavenging system[1] consisting of 0.8% D(+)glucose, 16500 units/ml glucose oxidase, 217000 units/ml catalase and 0.1% β-mercaptoethanol was added in this final washing buffer to stabilize the Cy3 and remove any reactive species.

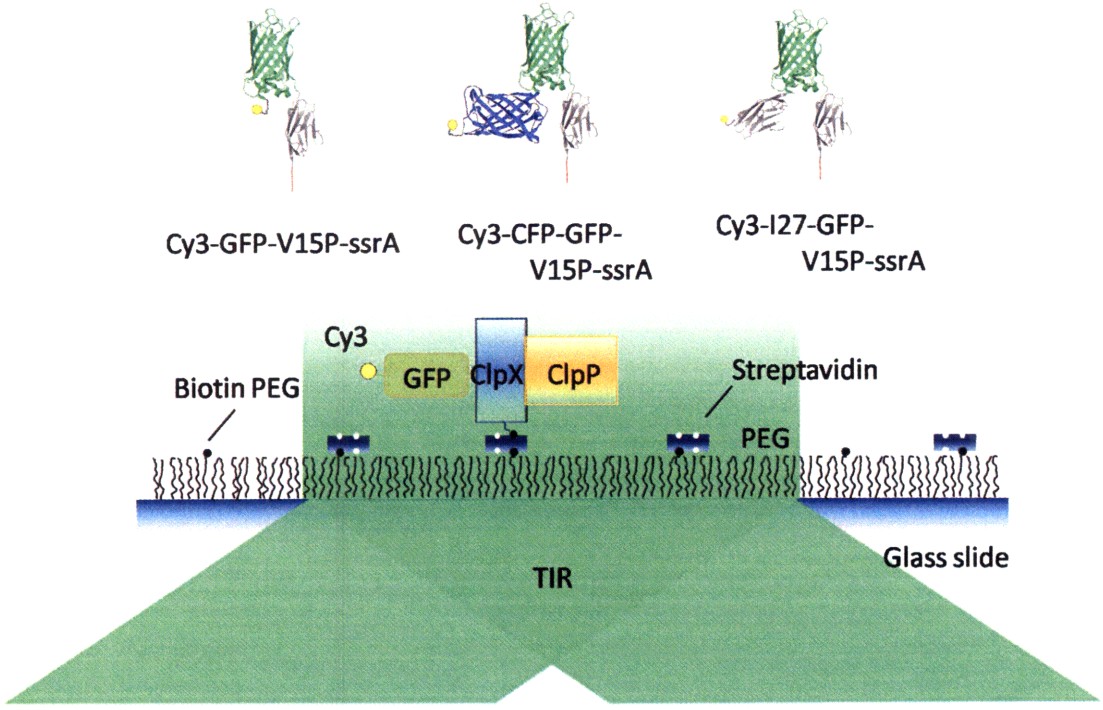

Figure 2-3: Schematics of the single molecule fluorescence assay. Three different types of substrates, labeled with Cy3 at the N-terminus, are used to monitor the kinetics of interaction between ClpXP and its substrate. Pre-engaged substrate-ClpXP complexes are immobilized on a PEG-coated glass surface in the presence of ATPγS, and flowing ATP and Mg^{2+} into the flow chamber results in the unfolding, translocation and subsequent degradation of remaining GFP tail region, which is being monitored by wide-field TIRF microscopy. Three substrates which consist of ssrA recognition tag (red), titin (gray), GFP (green), CFP (blue) and Cy3 (yellow) are displayed.

Chapter 3

Results

3.1 Design of ClpX Motor and Substrates

ClpXP degradation of substrates involves a series of events such as recognition, unfolding, translocation, degradation and product egress. The duration of these whole degradation processes is highly dependent on substrates, but in general it is not a subsec level. At least, it take several seconds up to several minutes. Thus, in order to visualize the kinetics of ClpXP degradation process at single molecule level, it is necessary to design a experiment with which we can monitor the activity of individual ClpXP stably interacting with associated substrates. This motivated us to design ClpX unfoldase and substrates such that ClpX were covalently crosslinked [38] and biotinylated at C-terminus and substrates were engineered to have GFP domain following I27 domain of titin and ssrA degradation tag. This particular titin domain contains a V15P mutation that makes it easier to denature.[29] Moreover, it can be unfolded by ClpX in the presence of ATPγS. On the contrary, it is found that ClpX cannot unfold GFP in the presence of ATPγS.[8] Partially degraded 38 residues reaching from the entry of ClpX pore to the ClpP active sites reduces the probability of dissociation of the substrate from the enzyme.[40] In this way, the ClpX was able to be immobilized on the surface while maintaining its functional form associated with ClpP and any free substrates on solution can be removed once the stable pre-engaged substrate-ClpXP complexes were formed in the presence of ATPγS. This strategy

allowed us to reach single molecule level without any dissociation of complex or increasing any background signal.

Three substrates were engineered : Cys-GFP-V15P-ssrA (single construct), Cys-CFP-GFP-V15P-ssrA (double construct) and Cys-I27-GFP-V15P-ssrA (construct with high mechanical stability). Introduced cysteine at the N-terminus of GFP was used to label substrates with a Cy3-maleimide, which is a exceptionally bright and stable organic dye used popularly in many single molecule fluorescence experiments.[7] Since the ssrA tag is a C-terminal degradation tag, the positioning of Cy3 at N-terminus of substrates allowed that it is the last element of the substrate that comes into contact with the pore of ClpX. Thus, fluorescence signal from Cy3 can be used to monitor the kinetics of substrate unfolding, translocation, degradation and even the product egress from ClpP degradation chamber.

3.2 Detection of Single Molecules

In the presence of saturating levels of ATPγS, the single molecule fluorescence assay described in previous section results in multiple individual fluorescent spots per field of view corresponding to specific ClpX-substrate interactions immobilized on a surface (Fig. 3-1). The size of illuminated region on the sample plan was around 15μm $\times$ 15μm and the typical number of fluorescent spots per field of view was around 100. After capturing digital images of the surfaces, a custom MATLAB software algorithm can be used to quantitatively identify them, determine their relative locations (Fig. 3-1), and track them over time of measurement. This information can then be used to determine how long it takes to observe the loss of each fluorescent signal of Cy3 molecules which can result from different circumstances.

Prior to analyzing the results, it is imperative to prove that the detected spot-like fluorescence images are indeed derived from single molecules. To verify this, several characteristic features typically seen for a single molecule must be examined. The clearest charateristics is the exhibition of single-step photobleaching.[19] Upon

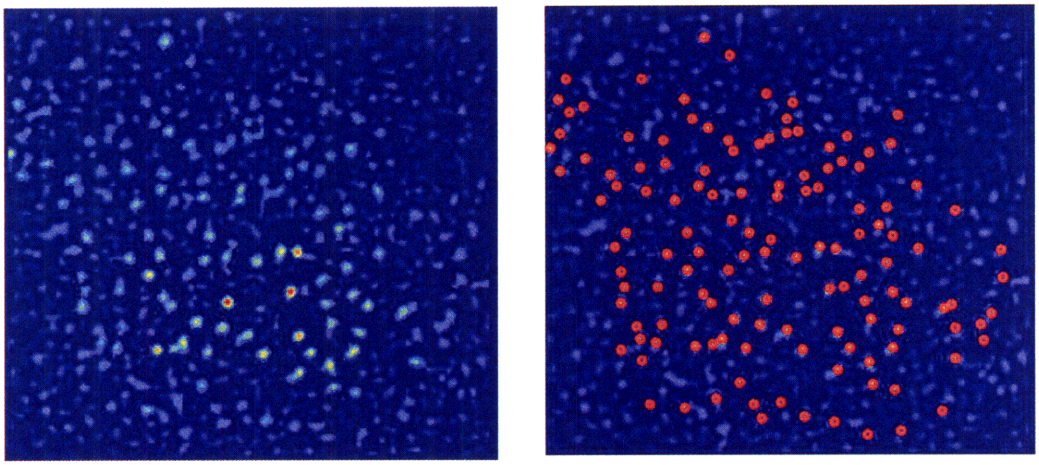

Figure 3-1: Detecting individual fluorescent molecules. (left) Representative image of a surface with immobilized ClpXP proteases engaging Cy3-labeled substrates. Each spot corresponds to an specific interaction between a single biotinylated ClpXP enzyme and a single Cy3-labeled substrate. (right) MATLAB code originally designed for particle tracking was used to process the image on the left. The software automatically identifies the single molecule fluorescence spots (red circles) and records their location. If a series of images are analyzed, this information can be used to track the longevity of Cy3 fluorophres and the result can be interpreted as Cy3 lifetime, degradation time constant of substrates by ClpXP, or any dissociation event depending on circumstances.

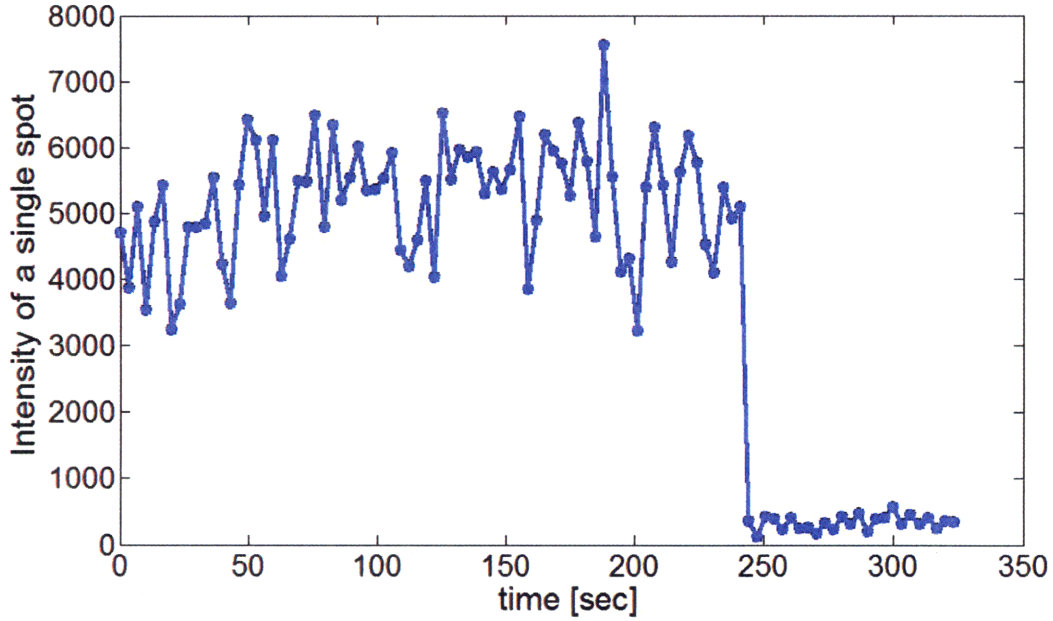

Figure 3-2: Proof that a fluorescent spot represents a single molecule. Intensity of a single fluorescent spot are tracked through the measurement and a single step photobleaching event which is characteristic of single molecule was recorded at round t = 250 sec.

continuous excitation, the fluorophore should photobleach based on Poisson statistics. The fluorescence intensity of the single dye molecule absorbed onto the PEG coated surface should show a single step descent, which was clearly seen in Fig. 3-2. In addition, fluorophore on the surface sometimes exhibited ON-OFF flickering, which is another charaterisitic feature of a single molecule fluorescence called blinking.[14]

3.3 Reduced Nonspecific Binding

In order to protect collected data from contamination, all signals in the image are supposed to come from the Cy3 fluorophores conjugated to substrates which are specifically engaged into ClpXP pore. However, it is always possible that some fluorescent particle or substrates nonspecifcally bound on the surface give similar shape of spots in the image, which makes those collected data worthless. Thus, it is always very important to obtain a surface that can allow for the only specific binding

of molecules of our interest in single molecule fluorescence experiments. Casein, predominant protein in cow milk, or Bovine Serum Albumin (BSA) were famous blocking agents and are still commonely used in many single molecule experiments mainly due to simple application.[31],[17] However, application of these surface passivation to ClpXP substrate degradation assay was not successful due to nonspecific binding between our substrates and these blocking agents themselves. Fig. 3-3 clearly shows that similar number of spots were still observed in the sample without any ClpX enzyme. Since biotinylated ClpX ATPase acts, in this single molecule assay, as a linker which connects Cy3 labeled substrates to the surface illuminated by TIR, this control experiment clearly indicates that those observed spots in the absence of ClpX were nonspecifically bound substrates. This initial failure in using conventional blocking agents motivated us to adopt polyethylene glycol (PEG) surface introduced in single molecule field recently.

PEG is particularly good at reducing protein affinity and has been used widely to suppress nonspecific protein binding in many single molecule experiments.[22],[34] The repulsive nature of PEG-treated surfaces has been attributed to steric effects arising both from the loss of conformational entropy of the polymer as the chains are compressed and from the energetically costly desolvation of the polymer by the impinging protein. Covalent attachment of bifunctionalized PEGs with a terminal N-hydroxysuccinimidyl (NHS) ester to amine-coated surfaces prevents desorption with time.[26] In our assay, 1% biotin-PEG-NHS was used in combination with mPEG-NHS to allow for the immobilization of biotinylated ClpX enzyme on the surface. Fig. 3-3 shows great improvement by using PEG-coated surface to suppress the nonspecific binding between substrates and glass surface. The comparable low number of spots was observed in a sample without any nucleotide (data not shown), indicating that labeled substrates were indeed bound on ClpXP in the ATP-dependent manner. Thus, we are now able to ensure that observed fluorescent spots in the image can be regarded as the light emitted from Cy3 molecules specifically engaged into the surface-bound enzyme.

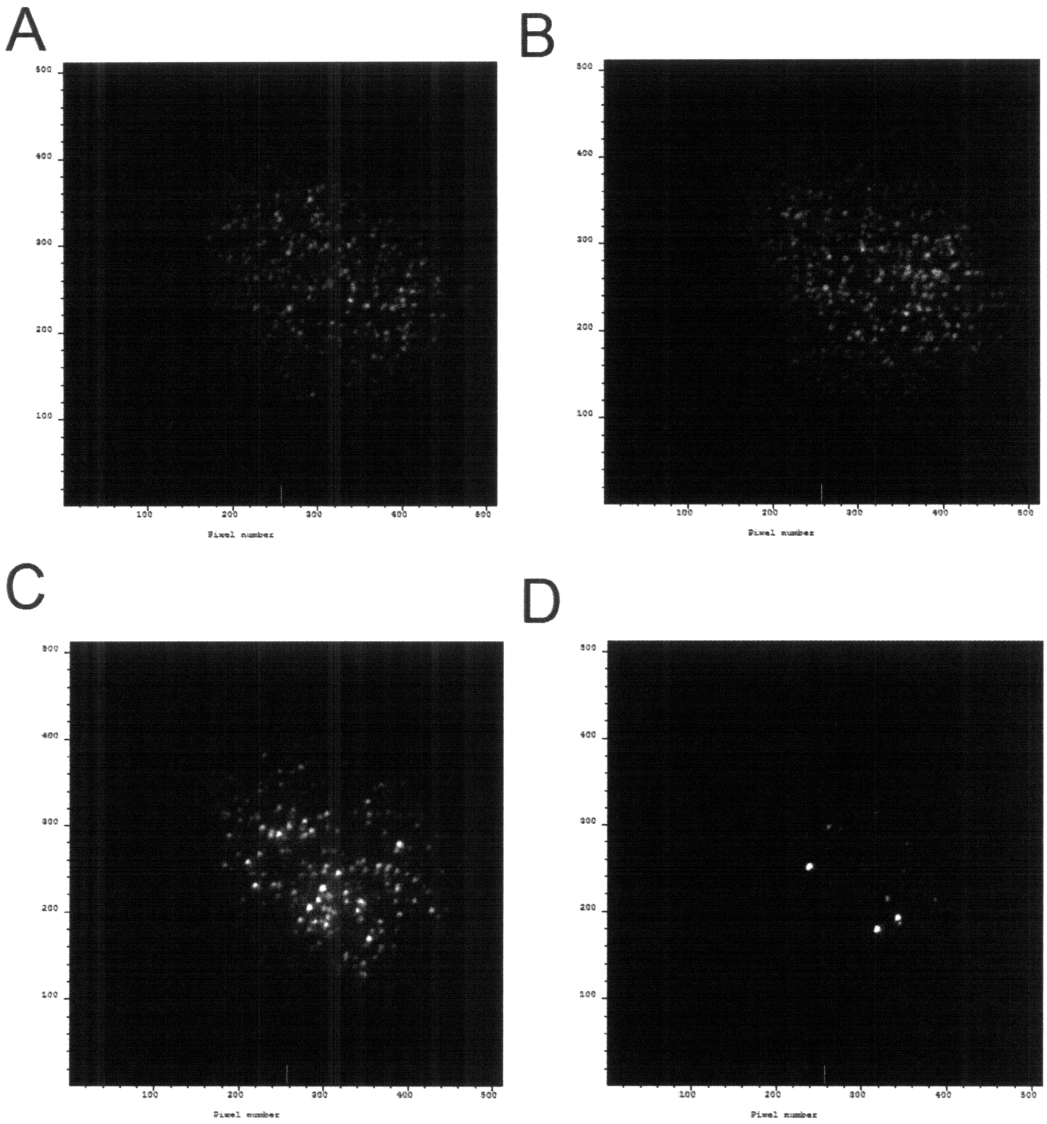

Figure 3-3: Reduced nonspecific binding with PEG surface. The EMCCD images of samples with ClpXP (A) and without ClpXP (B) incubated with Cy3-GFP-V15P-ssrA in the prescence of ATPγS on the casein surface. The existence of comparable number of fluorescent spots in the sample without ClpXP indicates the high degree of nonspecific binding of substrates. The images of samples with ClpXP (C) and without ClpXP (D) incubated with Cy3-GFP-V15P-ssrA in the prescence of ATPγS on the PEG surface. The nonspecific binding observed in the casein surface was almost removed by switching to PEG surface.

3.4 Pre-engaged Substrate and Buffer Exchange

A point-like fluorescent spot in optical microscopy produces an image that has the shape of an Airy disk due to the spectral filtering effect of limited physical size of aperture in every optical system. The width of this diffraction-limited image of fluorescent molecules scales as $\lambda/(2$ N.A.) where N.A. is the numerical aperture of the collection lens. If there exist too many immobilized fluorophores on the TIR-illuminated surface, it is impossible to dissect the position of individual molecules as well as to follow the signal of each fluorophores due to the light leakage from the other ones. Thus, it is critical to decrease the concentration of immobilized molecules of interest such low that each single molecule can be indentifiable as individual spots in the image. This makes it difficult to develop a single molecule fluorescence assay for probing the interaction between ClpXP and substrates since the affinity of ssrA degradation tag to ClpX is around 1μM which is too high concentration for single molecule level imaging.

We used a strategy to pre-engage substrates, containing nondegradable GFP in the prescence of ATPγS, into ClpXP before dilution to single molecule level. This allowed us to make stably bound fluorophores on the surface, and we can prevent the increased background which might be induced by any free Cy3-labeled substrates in a flow chamber. In order to form pre-engaged substrate-ClpXP complexes, engineered substrates were pre-incubated with ClpXP and ATPγS for 45min at 30°C. The complexes were then introduced into the flow cell and immobilized on the glass slide coated with PEG, through a biotinylated ClpX and PEG bound streptavidin linkage. In the presence of saturating levels of ATPγS, this single molecule fluorescence assay results in multiple, typically 100, individual fluorescent spots per field of view corresponding to specific ClpXP-substrate interactions immobilized on a surface.

These pre-engaged substrate-ClpXP complexes in the presence of ATPγS showed stability, consistent level of spots/field-of-view as indicated in Fig. 3-4. Before triggering degradation of substrate by ClpXP, we sequentially washed flow chambers with a series of buffers to exchange ATPγS with ATP and ready the system for degration.

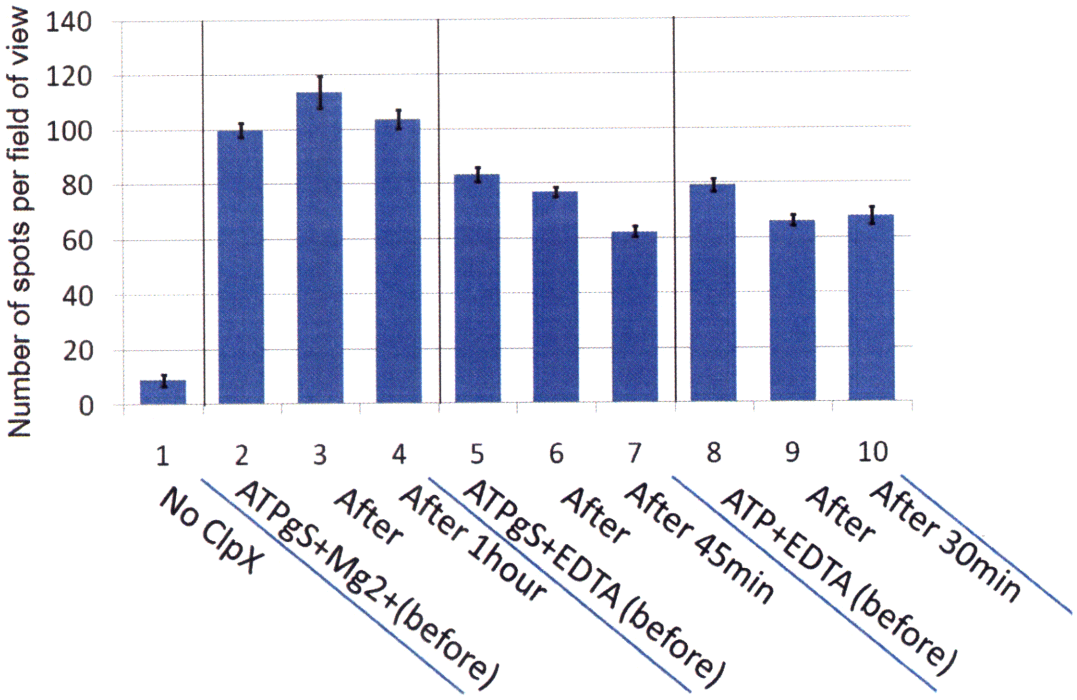

Figure 3-4: The behavior of surface-bound spots on the inflow of three different buffers. The number of spots per field of view was measured in different sample condition. The first sample without ClpX showed the low level of nonspecific binding of Cy3-labeled substrate on PEG coated surface. When three different types of buffers were flowed into the sample chamber, the binding between ClpXP and substrates labeled with Cy3 were observed to be stable enough to be unperturbed. Error bars are drawn with standard deviation error of each data column.

The first buffer contained 50mM EDTA and 2mM ATPγS to chelate Mg^{2+}. Then, the second buffer with 6mM EDTA and 1mM ATP (plus regeneration system) was added into flow cells to replace bound nucleotides from ATPγS to ATP. The Fig. 3-4 show the most of bound molecules on the surface remained at the same place even after a series of washing processes. Thus, at this point, ATP bound ClpX was expected to maintain association with the substrate but nucleotide hydrolysis was prevented due to lack of free Mg^{2+}.

3.5 Fluorophore Longevity

In the previous session, it was shown that pre-engaged substrate-ClpXP substrates were stably bound on the glass surface in the prescence of ATPγS. These complexes allowed for measuring longevity of Cy3 conjugated at the N-terminus of our engineered constructs. To test for photobleaching timescales, we measured the decay of the total number of Cy3 labeled GFP in the presence of ATPγS, using our standard image acquisition of capturing an image every 3 seconds. In other words, the fluorescence excitation laser irradiates the sample with a 300 ms pulse while the EMCCD camera simultaneously captures an image of the fluorescent signals from the irradiated surface. In between laser pulses, the fluorescence excitation laser is shuttered and the camera is idled for an adjustable dead time period (here 3s). Using this modulation, the tremendous increase in Cy3 longevity was obtained.[6] This increased fluorophore longevity may be attributed to the fact that the probability of incident of intersystem conversion, regarded as the main source of photobleaching phenomenon, is purely dependent on the number of excitation cycles that the molecule has experienced. Since the fluorophores are excited less often in the case of laser modulation, the probability of the onset of photobleaching is decreased.

Since the time scale of activities studied in this experiment using Cy3 probe was much longer than the excitation cycle used, this modulation of excitation laser light increased the longevity of fluorophores without failing to capture any biochemical activity of interest. Since the dissociation of Cy3-labeled substrates from the ClpX

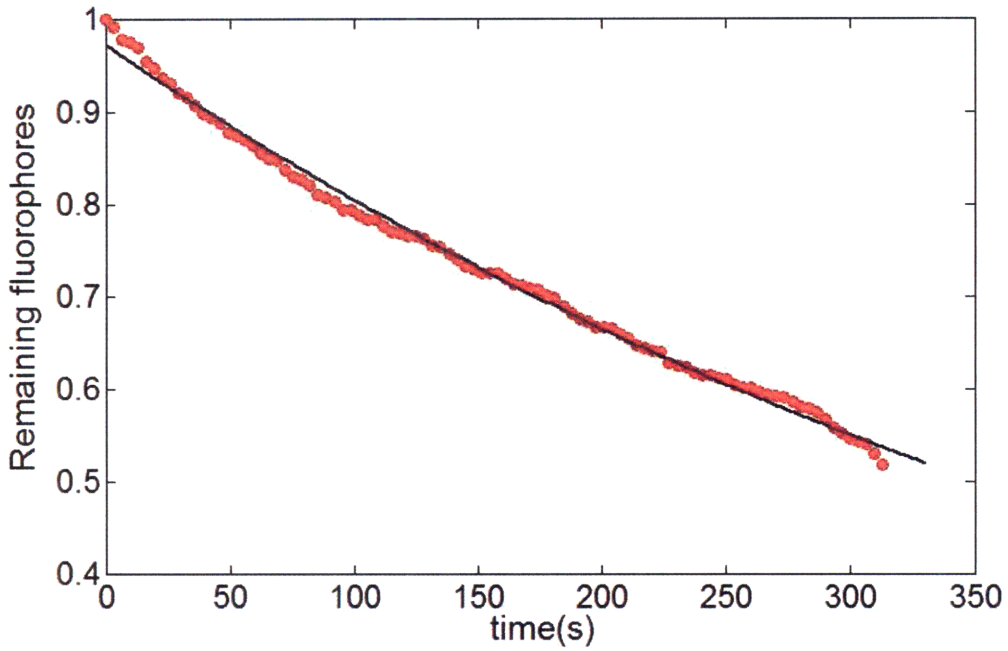

Figure 3-5: The longevity of Cy3 molecules attached to N-terminus of substrates with modulated illumination. The decay of the total number of fluorophores at each frame were plotted and fitted to a single exponential. Existence of oxygen scavenging system and 3 sec of illumination-dead-time between 0.3s of excitation period increased the longevity of Cy3 tremendously, ensuring a separation of timescales for measuring the degradation of substrates by ClpXP.

were observed to be minimized in the presence of ATPγS,(Fig. 3-4) the loss of individual signal can be interpreted as photobleaching. Cy3 molecules photobleached in a single exponential decay with a time constant of 347s.(Fig. 3-5) Cy3 photobleaching timescales were long enough to ensuring a separation of timescales for measuring the degradation of our Cy3 labeled substrates by ClpXP in the presence of ATP. In all measurements, an oxygen scavenging system[1] was used in the final buffer to increase the longevity of the fluorophores, and the intensity of excitation laser was maintained at a low level just above where individual spots can be still distinguishable.

3.6 Bulk Data

In various single molecule fluorescence assays, the surface immobilization of biological molecules on a surface without compromising their structure and function was tried and also verified.[57],[16],[46] In order to check if the surface immobilization of biotinylated ClpX on PEG surface did not perturb the function of ClpXP, two types of ensemble measurements in similar conditions with single molecule assay were conducted at room temperature in similar manners described in [40]. At first, the unfolding rates of GFP by ClpXP was determined using a stopped-flow technique.[40] Pre-engaged GFP-ClpXP complexes, similar with the one used in single molecule assay but without Cy3 molecules, were generated in the presence of ATPγS. When ClpXP-ATP-GFP complexes were mixed with Mg^{2+} in the stopped flow manner, ATP hydrolysis resumed and native GFP fluorescence was lost single exponentially with a time constant of 26s. When compared with the same experiment at 30°C, the unfolding slowed down about 5 times. In the second ensemble experiment, single turnover ClpXP degradation of a CFP-GFP-titin^{CM}-ssrA fusion protein was assayed to anaylze rates of both GFP unfolding and translocation. With this substrate, ClpX denatures GFP and must then translocate roughly 240 aa before unfolding CFP. A lag of 32s was observed between the loss of GFP and CFP fluorescence, representing the time necessary for GFP translocation and CFP unfolding by ClpX. ClpX unfolds

CFP as fast as it unfolds GFP.[40] Thus, ensemble measurements indicate that ClpX unfolds GFP with the time constant of 26s and then translocated unfolded GFP with the time constant of 6s.(For detailed analysis, see [40])

3.7 Substrate Degradation and ATP Dependence

Developed single molecule assay was used to monitor the ClpXP degradation of pre-engaged substrates. Pre-engaged substrate-ClpXP complexes were prepared as described in previous sections. After buffer exchange with EDTA and ATP to chelate Mg^{2+} and exchange bound nucleotides from ATPγS to ATP, the flow cell was mounted on sample plane of microscope capable of TIR imaging. After focusing and selecting a field of view, ATP and Mg^{2+} were injected into the flow cell to allow for ATP hydrolysis and initiate the ClpX unfolding of GFP. Image acquisition was triggered every 3 seconds and a custom software algorithm was used to quantitatively monitor the residence time, number of images, through which each spot remained. This spot lifetime indicated the time required by ClpXP to unfold, translocate and degrade our substrates in the presence of ATP.

Upon introduction of saturating ATP and Mg^{2+}, the substrate population showed an inital flat region followed by a rapid drop in fluorophore population with a long tail suggesting multiple kinetic steps. Overall decay of population was much faster than that observed in the presence of ATPγS, which indicates the ATP driven ClpXP degradation of remained tail region following GFP. Typically 10 percent of labeled substrate remained at the end of the 5 minute measurement suggesting a high percentage of motors were active. The shape of both population decay (Fig. 3-9) and spot lifetime (Fig. 3-6) suggests more than one rate limiting step is involved in the process. As expected from the extra CFP domain in Cy3-CFP-GFP-V15P-ssrA, CFP-GFP degradation showed a slower decay than GFP degradation. This fact can be more clearly identified through the spot lifetime data where the peak for CFP-GFP shifted to about twice that of GFP.

It is important to consider the origin of the signal in both single molecule and

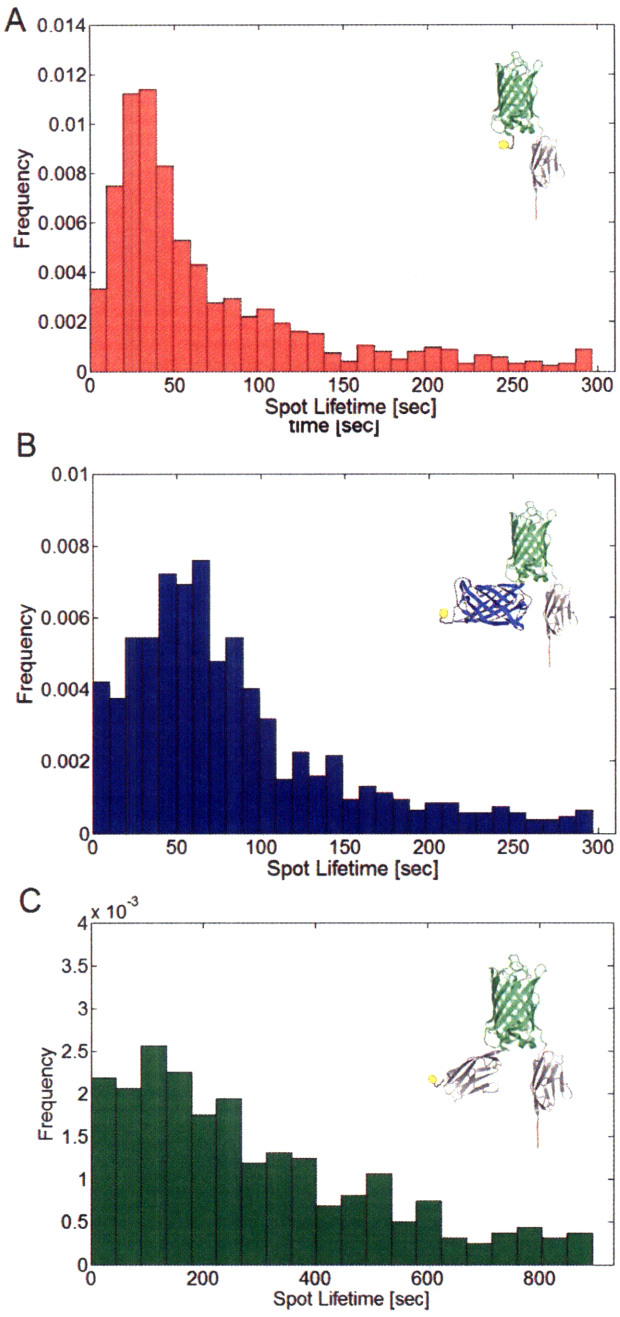

Figure 3-6: Engineered substrate degradation measured with single molecule assay. Triggering ClpXP degradation of substrates with ATP (1mM with regeneration system) and Mg^{2+} showed multiple rate limiting steps in degradation pathway for both GFP (A), CFP-GFP (B) and I27-GFP (C). Each graph shows the probability density function of the lifetime of individual spots, and substrates used in each measurement were shown inside graphs. The peak for CFP-GFP construct was shifted to about twice that of GFP, consistent with the fact that it has extra CFP domain. In the I27-GFP degradation, the illumination dead time between each excitation was increased to 9s while maintaining the total sum of exposure time.

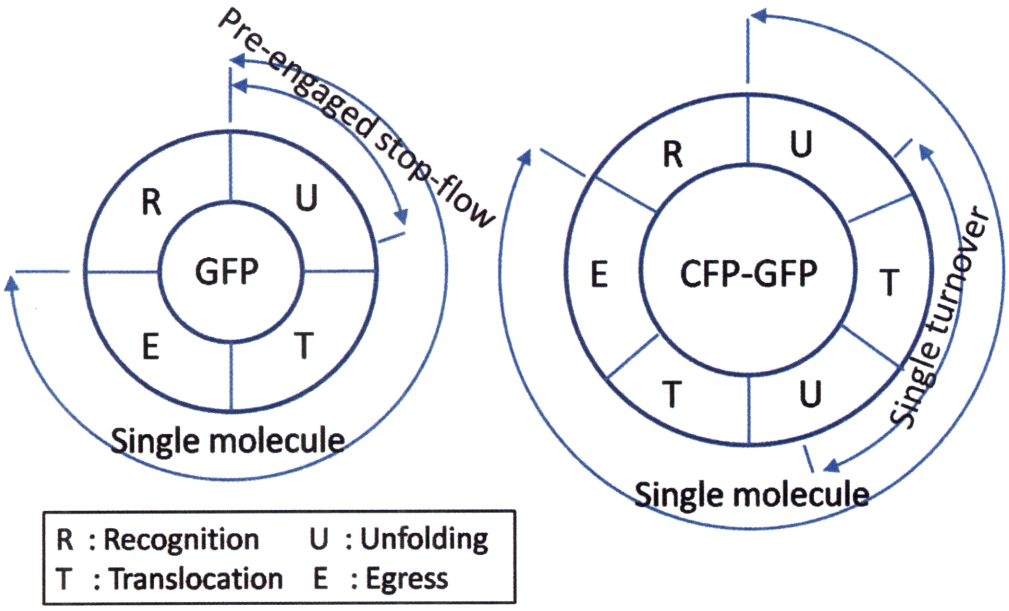

Figure 3-7: The difference between single molecule and ensemble measurement. In single molecule assay, the whole degradation kinetics from unfolding to translocation to product egress was measured, but stopped flow ensemble measurement for pre-engaged substrate was used to monitor only the unfolding of GFP by watching the loss of GFP fluorescence. In bulk single turnover degradation assay, the loss of both GFP and CFP was monitored and the time delay between these traces was regarded as the time to unfold and translocate a single GFP.

previously decribed bulk measurements because the single molecule measurement monitors fluorescence from a dye on the terminal location of the substrate and the bulk monitors an event where GFP is no longer a competent fluorophore.(Fig. 3-7) To further test this single molecule fluorescence assay, we engineered a substrate with an extra titin domain that degrades about five times slower than GFP in bulk.[40] Because of the slower decay, the time between each image was extended from 3 to 9 seconds while maintaining the same exposure time per image and equivalent overall exposure to excitation light. The degradation of titin-GFP, exhibiting much longer spot lifetime than that of CFP-GFP, showed consistency again with bulk.(Fig. 3-6)

Substrate degradation also slowed in the presence of both low concentrations of ATP and in the presence of low concentrations of ATPγS mixed with ATP.(Fig. 3-8) Rapid substrate release occured when fresh buffer with no nucleotide was introduced. The release was well described by a single exponential decay with a time constant of 13.6s. This rapid release may be due to opening of the hexameric ring of ClpX. The measurement indicates that the pore loop of ATP-bound ClpX subunits maintain an active grip on substrates to prevent their dissociation from ClpX.[39]

3.8 Kinetic Models

Our first two substrates are related by an extra CFP unit which allows for construction of kinetic models where parameters can be linked with respect to degradation to both our single and double constructs. Thus, our analysis includes a series of models with global fits of such parameters to maintain consistency between single and double measurements. In addition, we consider GFP and CFP here to have similar kinetics because CFP was shown to unfold and translocate with a time constant comparable to that of GFP unfolding and translocation [40]. Finally, all of our fits include a population of spots that photobleach where we have fixed the time constant to k_{pb}=0.003/s. We began by considering processing of the short substrate through unfolding and translocation steps represented by a two step three state process. In the case of our double construct, after the degradation of GFP, a second pair of unfolding

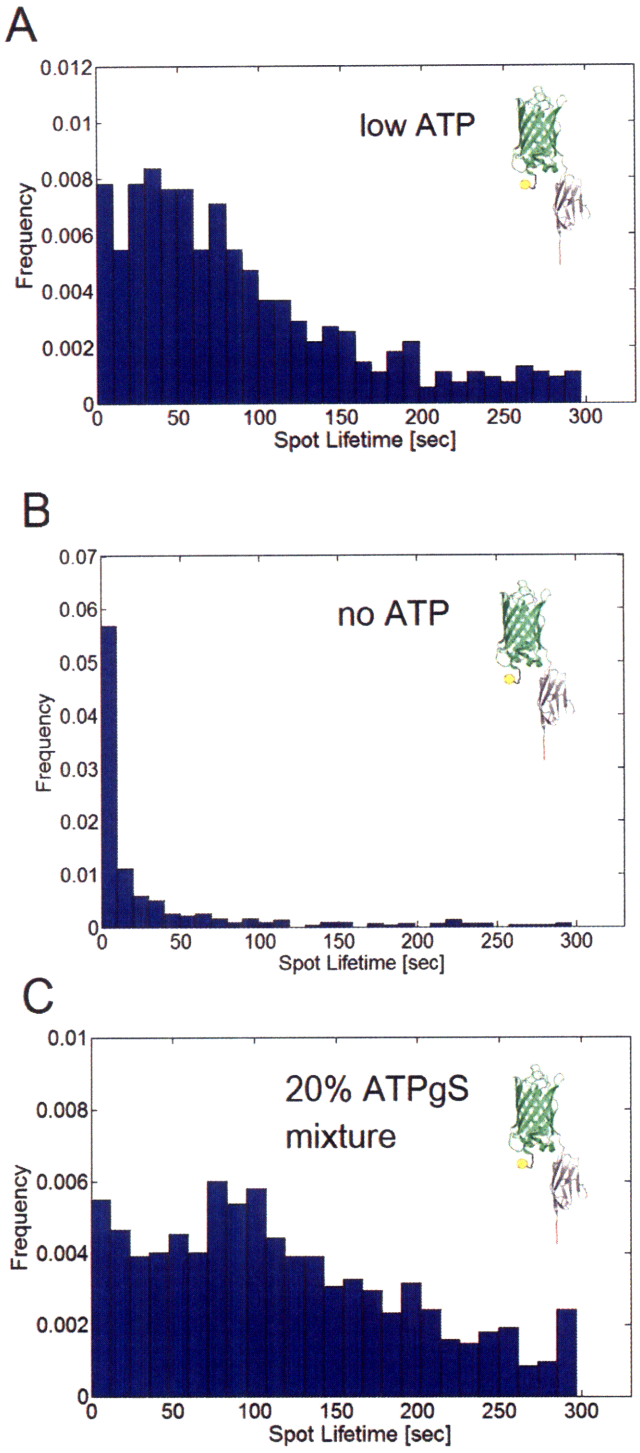

Figure 3-8: ATP dependence of ClpXP degradation of Cy3-GFP-V15P-ssrA. (A) The degradation slowed in the prescence of low nucleotide, 100μM of ATP. (B) In the absence of any ATP, the fast decay indicated the rapid release of bound substrate from ClpXP pore. (C) When ATPγS, the ATP analog which hydrolyze much slowly, was mixed with ATP, the degradation again slowed down.

and translocation steps occur for CFP for series processing of the double substrate in a fully sequential manner. :

$$\begin{aligned} &\mathrm{A}_1 \xrightarrow{k_1} A_2 \xrightarrow{k_2} A_3 && \text{GFP} \\ &\mathrm{A}_1 \xrightarrow{k_1} A_2 \xrightarrow{k_2} A_3 \xrightarrow{k_1} A_4 \xrightarrow{k_2} A_5 && \text{CFP-GFP} \end{aligned} \tag{3.1}$$

Although the fits to this fully sequential model, equation 3.1, were reasonable when applied individually, simultaneous global parameter fits to both curves failed to capture the kinetics and suggest that fully sequential model is not appropriate.(Fig. 3-9) As can be seen in Fig. 3-9, the initial region of this model fails to fit the degradation data severely. Thus a model consisting of sequential unfolding and translocation does not describe our data.

$$\begin{aligned} &\mathrm{A}_1 \xrightarrow{k_1} A_2 \xrightarrow{k_2} A_3 && \text{GFP} \\ &\mathrm{A}_1 \xrightarrow{k_1} A_2 \xrightarrow{k_1} A_3 \xrightarrow{k_2} A_4 && \text{CFP-GFP} \end{aligned} \tag{3.2}$$

Since translocation of an unfolded polypeptide is quicker than unfolding, we might assume for the double substrate that the first translocation occurs while the second unfolding step is underway or that these can be captured by a single exponential time constant.(Equation 3.2) In addition to binding, denaturation and translocation of substrate by ClpXP, and based on the experiment showing the difference between predicted single-turnover degradation time and observed steady state,[40] additional

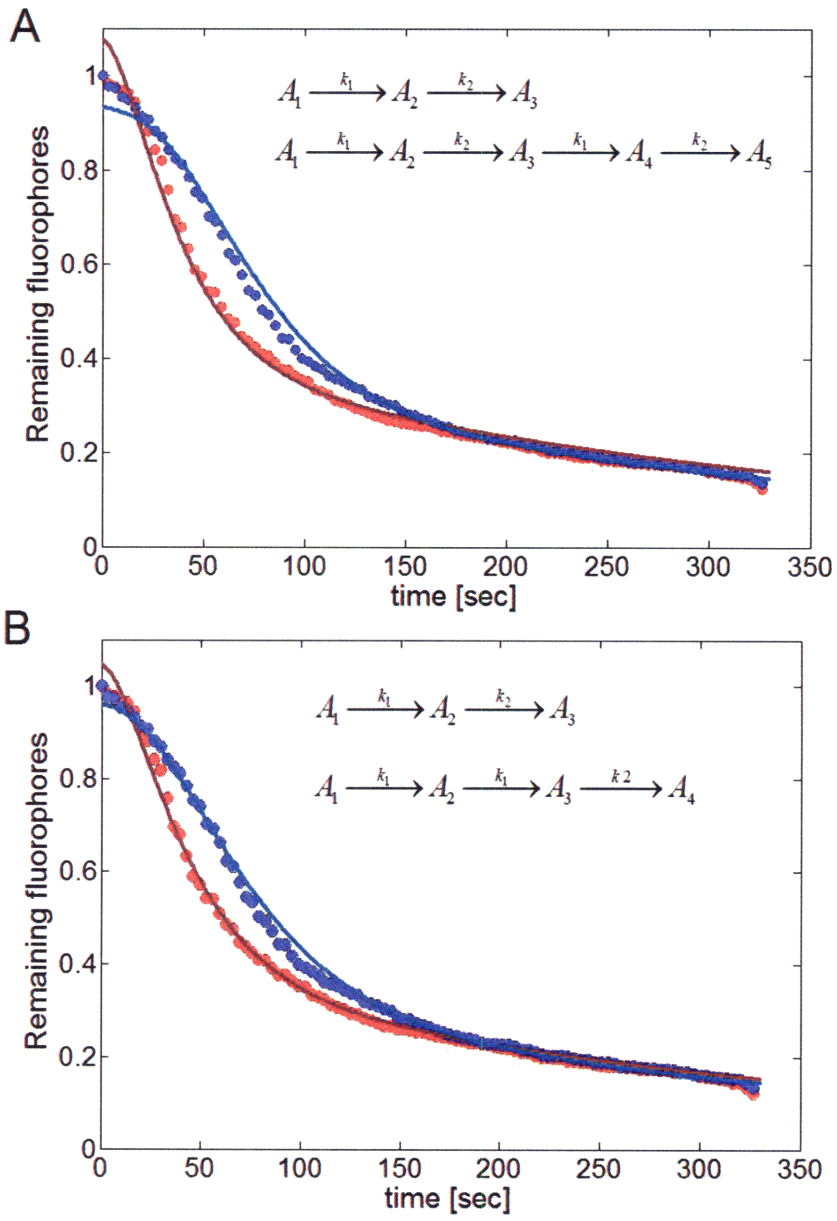

Figure 3-9: Simulataneous global fits of the kinetic models for ClpXP degradation. The probability density functions of each individual spot for the degradation of both substrates were converted to these types of total population decay graph to fit different types of kinetic models. (A) The severe discrepancy observed at the beginning of fits indicates that fully sequential model (Equation 3.1) may not be appropriate to describe the ClpXP degradation. (B) When only single extra parameter k_1 was added for the double construct,(Equation 3.2) the fits were greatly improved.

steps such as termination of a substrate-processing cycle or product egress from ClpP may also contribute to the observed kinetics. Since the loss of fluorescence signal in our single molecule assay occurs when Cy3 molecules are egressed from ClpP, our results may show this egress of product or any termination process in a direct way. Thus, after the degradation of terminal protein domain with kinetic rate of k_1, our second model includes an additional process that terminates the substrate processing with kinetic rate of k_2. Since translocation of an unfolded polypeptide is quicker than unfolding, the unfolding and translocation is assumed to be described in single exponential kinetics.(Equation 3.2) Thus our second model includes the same form for the single substrate equations but a simpler form for double. For this model, the decay of both substrates was well fit globally with kinetic rates,(Fig. 3-9) suggesting a longer terminating process k_2 occurs once at the end of processing of each substrate.

Chapter 4

Discussions and Future Directions

Developement of the single molecule fluorescence assay has enabled probing the kinetic details of interaction between ClpXP protease and its substrates. A covalently crosslinked ClpX hexamer maintained oligomerized at the nM level concentrations required for the single molecule measurement. Surface, and immobilization of ClpXP complexes through biotin-streptavidin linkage provided an opportunity to follow the kinetics of degradation activity of individual ClpXP molecules. Cy3-labeled engineered substrates containing nondegradable GFP in the prescence of ATPγS formed stable pre-engaged substrate-ClpXP complexes where the whole substrate degradation pathway, from unfolding to egress of degraded products, could be monitored without competing with dissociation or additional background characteristic of free labeled substrate in solution. The combined single molecule and ensemble approach in this study showed directly an additional process that ClpXP encounters at the end of degradation of single substrate. Most previous studies on ClpXP protease were based on ensemble measurements where the final termination processes of degradation was hard to be addressed.

In previous ensemble studies, translocation of substrates through the ClpX pore is envisioned to be a dynamic process in which a translocating polypeptide is pulled through the pore by the conformational changes during ATP hydrolysis in the AAA+ enzyme but can also slip back and even dissociate completely from the hexamer.[40] A

fusion protein with three repeated titin domains showed partitioning between unfolding and release in the saturating concentration of ATP.[30] In other study with GFP domain, ClpX-bound GFP was suggested to partition between dissociation and denaturation with altering partition coefficients depending on the length of the degradation tag and attached polypeptide segments.[40] In single molecule experiments described above, however, most of the engaged GFP tail domains in the presence of ATPγS were stably bound on the surface at least for an hour.(Fig. 3-4) Even in the absence of any nucleotide hydrolysis due to the lack of Mg^{2+}, ClpX-bound substrtates showed stability. Hence, this result needs further study but it is possible that the pore loop of each ClpX subunit that interacts with polypeptide may show different affinity for unfolded substrates depending on the nucleotide state of subunits. ATP-bound subunit might tightly bind the polypeptide and prevents slippage.

Although it depends on the type of substrate, it has been regarded that denaturation and translocation are the most critical rate limiting steps in the proteolysis of typtical native proteins.[29] Substrate degradation and release of substrates were expected to take place fast based on the studies showing the size distribution of peptide products is independent of the rate of translocation.[12],[50] In my experiment, the decay of total number of fluorophores after addition of ATP and Mg^{2+} couldn't be explained without thinking of an extra step, besides unfolding, translocation and cleavage, that Cy3 conjugated substrates should go through.(Fig. 3-9) It is not fully known what is the exact nature of this final process but the product egress can be one of them. If that is the case, this controversial result may be attributed to the fact that the Cy3 molecule was conjugated at the opposite end of substrates with the ssrA degradation tag. Since the Cy3 molecules were delivered to the degradation chamber of ClpP lastly, there might be no peptide fragment that could push Cy3 to go out of the chamber and diffuse away.

With the developed single molecule assay for ClpXP, it is now possible to observe the enzymatic activities of individual degradation machinery, ClpXP protease.

Observation of the strong conservation of several important motifs and architectural homologies among AAA+ unfolding machines suggest that all of these enzymes will operate by a similar mechanism.[40] The single molecule study of ClpXP as a model enzyme for AAA+ protein will provide invaluable informations on how these molecular motors convert energy and accomplish given tasks.

Appendix A

Protocols

Etching glass coverslips

Surface passivation with polyethylene glycol (PEG)

Labeling with Cy3

Glass flow cells with drilled holes

Single molecule fluorescence assay for ClpXP

A.1 Etching glass coverslips

(Adapted from Polly Fordyce)

Etching coverslips removes the wax layer on the glass, enhances adhesion, and reduces back-ground fluorescence.

Materials

Reagents:

Potassium Hydroxide (KOH)

Ethanol (100%)

ddH2O (Millipore, Dedon Lab)

Equipment:

Corning Coverslips, 24 x 60 mm, 1 thickness (Cat 12-553-6, Fisher Scientific)

Teflon racks (custom made)

Procedure

1. Dissolve 100 g of KOH in 300 mL of 100% ethanol in a 1 L beaker. Stir with a stir bar until KOH is completely dissolved or for 30 min.

2. Place coverslips in Teflon racks. Usually we do 6-10 racks per procedure.

3. Fill another 1 L beaker with at least 300 mL of 100% ethanol and two additional 1 L beakers with at least 300 mL of ddH2O. Degas all four beakers (two at a time) in the bath sonicator (degas setting) for 5 min. After degassing, place one of the ddH2O beakers and the KOH beaker in the bath sonicator.

4. Submerge one coverslip rack in the KOH solution and sonicate for 5 min.

5. Wash coverslips by dipping the rack up and down or spinning it in the ethanol beaker.

6. Wash coverslips by dipping the rack up and down or spinning it in the ddH2O beaker.

7. Submerge the rack of coverslips in the ddH2O beaker in the sonicator and sonicate for 5 min.

8. Spritz coverslips with ddH2O bottle. Do each coverslip side at least twice.

9. Spritz coverslips with ethanol bottle. Do each coverslip side at least twice.

10. Repeat steps 4-9 for other racks. Note that the ddH2O and KOH beakers in the sonicator can contain coverslip racks during sonication at the same time.

11. Dry rack in oven for at least 15 min at 100 oC. Store coverslips in racks inside sealed containers at room temperature. They last about a week.

A.2 Surface passivation with polyethylene glycol (PEG)

PEG is typically regarded as the gold standard in preventing nonspecific binding. This protocol describes a procedure for covalently coating etched coverslips with PEG. The PEG in turn can be functionalized with different moieties on which single molecule assays can be constructed. In particular, PEGylated surfaces resulting from this protocol will contain 1% biotin and are prepared in two steps. The first step coats etched coverslips with aminosilane and the second steps attaches the PEG molecules to the primary amines in the silane.

Materials

Reagents:

3-Aminopropyltriethoxysilane (Cat A3648, Sigma)

mPEG-SVA, MW 5000 (Laysan Bio)

Biotin-PEG-SVA, MW 5000 (Laysan Bio)

Acetone (Cat 154598, Sigma)

Buffers: 100mM Sodium bicarbonate, pH 8.7, filtered (fresh)

Procedure

1. Expose coverslips to plasma discharge with power setting at "high" for 3 4 minutes, opening the pump valve to bleed in a small amount of air and increase the plasma intensity about every minute. (If you have coverslips just etched with KOH before PEGylation, you can use them without additional plasma etching.)

2. Submerge coverslip in 400ml pure acetone and incubate for 5 minutes. While incubating, mix 400ml of acetone with 8ml of aminosilane to make a 2% solution.

Mix the solution with the glass rod until the turbidity disappears.

3. In the meanwhile, take the mPEG and biotin-PEG jars and place them on the bench top. It is important to let them equilibrate to room temperature before use because they are very moisture sensitive.

4. Transfer coverslips to 2% aminosilane solution and incubate for 20 minutes at room temperature to allow the (3-aminopropyl) triethoxysilane to functionalize the coverslip surface. In the middle of incubation, sonicate the beaker with the coverslips for a couple of minutes.

5. Submerge coverslips in ddH2O, and gently dip for about 30 seconds to remove excess silanizer.

6. Individually dry each coverslip in a stream of particle-free compressed nitrogen or air.

7. Dissolve 80mg of the mPEG and 5mg of the biotin-PEG in 640ul and 40ul of freshly made sodium bicarbonate, respectively. Centrifuge at 7200rpm for a minute to remove bubbles. Add 6.4ul of the biotin-PEG solution to the mPEG solution, which makes 0.125% biotin-PEG in the 12.5% mPEG solution.

8. Place 80μl of mixed PEG solution on a amine-functionalized slip and make a sandwich with another functionalized slip using some spacer slips.

9. After 4 hours of incubation at room temperature, rinse slips with ddH2O and dry with compressed air.

10. PEG-coated slips are stable at least for a week when stored in dark vacuum.

A.3 Labeling Cys residues with Cy3 fluorophore

This protocol presents a simple method for labeling cysteine residues with Cy3 maleimides. In particular, the method labels a N-term cys in Cys-GFP-I27(V15P)-ssrA, making the ClpX substrate easier to track and detect in single molecule fluorescence experiments.

Materials

Reagents:

364 μM Cys-GFP-I27(V15P)-ssrA (courtesy of Andreas Martin)

Cy3-maleimide (Cat PA23031, GE Healthcare)

Buffers:

Labeling buffer (25mM HEPES, pH 7.2, 50mM KCl, 1mM EDTA, 10% Glycerol)

Equipment:

Desalting chromatography column (Cat Bio-Gel P-6 gel, BioRad)

Procedure

1. Dilute Cy3-maleimide monoreactive pack in 50 μL of DMF, which gives a Cy3concentration of about 3 mM.
2. Dilute protein to 75 μL 10 μM in labeling buffer and buffer exchange twice toremove DTT.
3. Add 2 μL of the Cy3-maleimide to the buffer-exchanged protein and incubate overnight. Cover tube with aluminum foil paper to avoid photobleaching. Note that optimal pH range for the cysteine-maleimide reaction is 7.0-7.5.
4. Remove excess or unreacted Cy3 by desalting the reaction mixture in three columns.

A.4 Glass flow cells with drilled holes

This protocol describes a method to construct a glass flow cell that can be used in single molecule fluorescence assay for ClpXP. Normal glass slides are drilled with a diamond bit and etched with KOH. These glass slides can be recycled after usage.

Materials

Reagents:

ddH2O

Ethanol (100%)

Potassium Hydroxide (KOH)

Equipment:

Glass slide

Drill

Diamond drill bit (1.6mm, UKAM)

Procedure

1. Drill two holes that are separed by around 4cm with diamond drill bit. While drilling, put a small drop of ddH2O on the position of holes as a coolant.
2. Check if drilled holes fit well with tubings that will be connected in measurements. If not, trim the holes manually with the drill bit.
3. Etch drilled slides with KOH in similar way desribed for coverslip cleaning. Store them in vacuum for future usage.
4. After usage, dip used slides in ddH2O for several days. Attached coverslips and double sticky tapes can be easily removed by wipping outff with a razor blade.

A.5 Single molecule fluorescence assay for ClpXP

This protocol describes a method for preparing an assay designed to probe ClpXP degradation of its pre-engaged substrates at the single molecule level using single molecule fluorescence. The experiments take place in a pre-drilled glass flow cell to facilitate the quick exchange of buffers prior to measurements taking place. All buffer exchanges, except the last one, take place with a pipetman and a vaccum manifold.

Materials

Reagents:

1 mg/mL streptavidin in PBT (Cat S000-01, Rockland)

200mM ATPγS in PD (Cat 10

20mM ATP in PD (Cat A7699, Sigma)

biotinylated ClpX

ClpP

Substrates

Creatine Kinase in PD

Creatine Phosphate in PD

β-mercaptoethanol

Glucose in PD

Gloxy in PD

Buffers:

PBT (100mM phosphate buffer, pH 7.5)

PD (25mM HEPES, pH 7.6, 100mM KCl, 10mM MgCl_2, 10% Glycerol, 0.1% Tween)

Equipment:

Glass flow cell

1mL syringe (Cat BD309602, VWR)

Needle connector, 18GA $\times$ 1/2" long (Cat 75165A675, McMaster-Carr)

Tubing, 0.07" OD, 0.04" ID, 0.015" thickness (Cat 63018-088, VWR)

Procedure

1. Premix substrates with 2mM ATPγS, 0.31μM ClpX and 1μM ClpP for 45 min at 30 °C.

2. Dilute streptavidin to 0.01 mg/mL in PBT and flow 20μL into a flow cell made with a pre-drilled glass flow cell and a PEG-coated coverslip. Incubate for 10 min and wash out with PD buffer.

3. After 45min of incubation, dilute the premixed solution into appropriate dilution to reach the single molecule level while maintaining ClpP and ATPγS concentration.

4. Flow 20μL of the diluted solution into flow cells and incubate for 20min.

5. Prepare 3 different types of washing buffers: 2mM ATPγS + 50mM EDTA in PD buffer without Mg^{2+}, 1mM ATP (with regeneration system) + 6mM EDTA in PD buffer without Mg^{2+}, 1mM ATP + PD buffer. 800nM of ClpP was maintained in these buffers, and oxygen scavenging system (0.8% D(+)glucose, 16500 units/ml glucose oxidase, 217000 units/ml catalase and 0.1% β-mercaptoethanol) was included in the final washing buffer.

6. Once the substrate incubation has transpired, wash flow cells with the first two prepared buffer sequentially. Incubate for 2 minuates between each washing step.

7. Using the syringe, suck enough final washing buffer to fill a tube, and connect the tubes on ports of the flow cell.

8. Place the fully assembled flow chamber on the microscope stage and hold it with clips to prevent any drift. Look for single molecule fluorescence spots using the EMCCD camera. Once a region of interest is identified, close all

shutters immediately and switch the Andor camera to external mode and set it to acquire images every 3s for 300ms. Start recording a movie of 100 images. As soon as the first image comes on screen, start pushing the washing buffer through the flow cell.

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