WATCHING BIOLOGICAL NANOMOTORS AT WORK: INSIGHTS FROM SINGLE-MOLECULE STUDIES

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

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To my Family

Mallavva and Laxmi Rajam Chada

Narmadha and Nagarjuna Chada

Hymavathi Mamindla

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ABSTRACT

Part 1: High resolution (≈ 1 nm lateral resolution) biological AFM imaging has been carried out almost exclusively using freshly cleaved mica as a specimen supporting surface, but mica suffers from a fundamental limitation that has hindered AFM's broader integration with many modern optical methods. Mica exhibits biaxial birefringence; indeed, this naturally occurring material is used commercially for constructing optical wave plates. In general, propagation through birefringent material alters the polarization state and bifurcates the propagation direction of light in a manner which varies with thickness. This makes it challenging to incorporate freshly cleaved mica substrates with modern optical methods, many of which employ highly focused and polarized laser beams passing through then specimen plane. Using bacteriorhodopsin from *Halobacterium salinarum* and the Sec-translocon from *Escherichia coli*, we demonstrate that faithful images of 2D crystalline and non-crystalline membrane proteins in lipid bilayers can be obtained on common microscope cover glass following a straight-forward cleaning procedure. Direct comparison between data obtained on glass and on mica show no significant differences in AFM image fidelity. Repeated association and dissociation of SecA with SecYEG indicated that the proteins remain competent for biological processes on glass substrates for long periods of time. This work opens the door for combining high resolution biological AFM with powerful optical methods that require optically isotropic substrates such as ultra-stable and direct 3D AFM. In turn, this capability should enable long timescale conformational dynamics measurements of membrane proteins in near-native conditions.

Part 2: In the second part of this work we studied SecA-ATP hydrolysis and catalase enzyme dynamics. Both of these protein macromolecules were observed to be highly dynamic during catalytic turnover. Single molecule studies of catalase indicated that the enzyme undergoes oligomeric state changes when exposed to H_2O_2 . Conformational dynamics of the SecA-ATPase was visualized at the single molecule level and the protein macromolecule flickers between a compact and expanded state in the presence of ATP, indicating reversible conformational changes. Future studies in the lab will shed more light onto these biological processes.

1. Introduction

Theoretical models describing observables arising from ensembles of molecules were established long before the first single molecule techniques came into existence. Hence, historically the development of laws in physical chemistry have employed the gas constant R which equals Avogadro's number times Boltzmann's constant k_B . This is why RT , energy per mole, has traditionally been expressed in units of kilojoules and kilocalories. In recent decades, this is no longer the case as revolutionary single molecule techniques carried out both *in vivo* and *in vitro* have been developed providing ways to observe biological processes which were once unattainable. Such measurements can reveal hidden subpopulations, heterogeneities, intermediates and individual molecular trajectories. In the past few decades, single-molecule techniques have become accepted and widespread. In several disciplines of science it has become natural to express thermal energy $k_B T$ in terms of $pN * nm$, instead of energy per mole.

Past decades have seen a great advance in single molecule methods. Such approaches open new avenues of investigation that were not possible using traditional techniques which measure average properties of molecular populations. This has opened doors to study and analyze biological systems at the single molecule level yielding new and important insights.

As an example, let us consider molecular motors. Although, traditional biological assays that studied motor movement support constant-velocity movements, it has been revealed by single molecule studies that many of them take discrete steps¹⁻⁴. 1986 was a

momentous year for the physics community. This year, nearly three decades, ago saw the first demonstration of two significant single molecule techniques, namely optical tweezers, which has demonstrated the discrete steps of molecular motors, and atomic force microscopy (AFM) that produced topographic images at atomic resolution^{5,6}. These two techniques developed by physicists, form a major backbone of current research in single molecule techniques for studying biological systems.

Atomic Force Microscopes with well-established protocols to study biological molecules are now commercially available. Standard protocols for single molecule studies using optical tweezers have also been established but for precise measurements they are usually still custom-built. Fluorescence techniques that are more familiar to biologists are now capable of measuring nanometer-scale distances and localizing individual biological molecules^{1,7,8}. Super resolution imaging methods paved a new way to visualize cell function in real time at single molecule level and routinely break the Abbe diffraction limit imposed by the wavelength of light in traditional optical microscopy.

The past decade has seen an enhancement in both lateral and time resolution for a variety of single molecule methods. Single-molecule fluorescence resonance energy transfer (smFRET) can attain resolution of as small as 3 – 5 Å with as little as 100 photons. High-speed atomic force microscopy has been used to unfold single protein molecule at an astonishing speed of millimeter per second which can directly compare to all-atom molecular dynamics simulations leading to an impressive agreement between molecular dynamics simulations and single molecule experiments⁹.

1.1. Hybrid approaches

‘Convergence’ is a new mantra in the single molecule biophysics community. Single molecule methods took a big leap ahead in the past ten years, pushing the envelope in precision and complexity to maximize the information content and allow direct access into living systems. A number of recent studies have described hybrid single molecule techniques (Fig 1.1)¹⁰. Bringing two complimentary single molecule techniques to bear on single macromolecular complex is clearly going to yield important insights in the near future.

Figure 1.1: Coupling two different complementary single-molecule techniques enables new capabilities that cannot be accessed using individual methods alone¹⁰.

Recently the Bustamante group developed a rotor-bead assay in which the associated length of DNA is directly visualized at the same time as the rotation of a fluorescent bead allowing them precisely determine the rotational pitch of DNA as it being inserted into a viral capsid by a packaging motor¹¹. Their experiments also revealed fine details of stepping by viral DNA packaging machine. Del Rico et al demonstrated a different type of hybrid instrument by combining magnetic tweezers with single-molecule fluorescence detection¹². They demonstrated that a cryptic binding site for vinculin protein can be exposed by applying physiologically relevant forces to talin protein, which bridges the cell membrane to the underlying cytoskeleton.

Although single molecule techniques continue expanding their scope with new techniques being invented, the most commonly used techniques that have received attention are optical tweezers, magnetic tweezers and atomic force microscopy (Table 1.1)¹³.

Table 1.1: Comparison of commonly used single-molecule force spectroscopy techniques

	Optical tweezers	Magnetic tweezers	AFM
Spatial resolution (nm)	0.1–2	5–10 (2–10)	0.1–1
Temporal resolution (s)	10^{-4}	10^{-1} – 10^{-2} (10^{-4})	10^{-6}
Stiffness ($\text{pN}\cdot\text{nm}^{-1}$)	0.005–1	10^{-3} – 10^{-6} (10^{-4})	10 – 10^5
Force range (pN)	0.1–100	10^{-3} – 10^2 (0.01 – 10^4)	0.5 – 10^4
Displacement range (nm)	0.1 – 10^5	5 – 10^4 (5 – 10^5)	0.5 – 10^4
Probe size (μm)	0.25–5	0.5–5	10–250
Typical applications	3-D manipulation Tethered assay Interaction assay	Tethered assay DNA topology (3-d manipulation)	Imaging, pulling and interaction assays
Features	Low noise and drift dumbbell geometry	Force clamp Bead rotation Specific interactions	High resolution imaging
Limitations	Photo damage Sample heating Non specific	No manipulation (Force hysteresis)	Large high- stiffness probe

1.2. Combining AFM with Advanced Optical Techniques

AFM, being commercially available, is one of the most widely used single-molecule manipulation techniques. The “gold standard” specimen support used in AFM is mica, known for its inherent flatness, cleanliness, hydrophilicity and biological compatibility. But mica suffers from biaxial birefringence that has hindered its integration of with many powerful optical techniques. Glass on the other hand, being optically isotropic, is an ubiquitous specimen support for advanced optical microscopy techniques¹. The topography of glass being much rougher compared to mica, is not considered a good specimen support for AFM as its roughness hinders one from achieving high resolution images of membrane embedded proteins. Hence in the first part

of the work described here we sought to develop protocols to optimize glass as specimen supporting substrate for atomic force microscopy which would enable us to integrate optical stabilization methods into AFM to gain new insights into processes involving biological macromolecules at work.

1.3. Biological molecules of interest

1.3.1. Sec-translocase: protein translocation nano-machinery of *Escherichia coli*

In cells from all three domains of life, protein trafficking across membranes is a ubiquitous and crucial phenomenon¹⁴. More than one-third of all proteins synthesized function in a membrane or outside of the cytoplasm. Hence large numbers of proteins have to translocate into or through at least one lipid membrane to reach their final destination. But lipid membranes are hydrophobic barriers. They are intrinsically impermeable to ions and polar solutes. Then, the question arises: how are proteins, which are synthesized in the cytosol, translocated to their final destinations across these hydrophobic barriers?

Millions of years of evolution have produced a remarkable array of mechanisms to translocate proteins across and integrate into hydrophobic cell membranes after their synthesis in the cytosol. Sixteen such systems have been discovered in bacteria alone^{15,16}. Of these sixteen discovered mechanisms, the Sec pathway is ubiquitous in all three domains of life.

The translocon SecYEG is a dynamic macromolecule embedded in the membrane that recognizes exported proteins at the membrane and catalyses their export. The functional core of the Sec translocon consists of a protein conducting channel that spans across the membrane. This complex is built of SecY, SecE and SecG polypeptides.

Several crystal structures of the protein conducting channel SecYEG at various resolutions, of both eukaryotic and prokaryotic origin and bacterial SecA motor, are now available¹⁷⁻³¹.

Protein export through the Sec pathway is a multi-stage event that occurs mostly post-translationally. An overview of posttranslational protein translocation through the Sec pathway is illustrated in Figure 1.2. Nascent pre-proteins are synthesized at the ribosome in the cytosol. These protein chains are recognized directly by piloting factors such as the SecB chaperone^{32,33}. The Sec-translocon cannot translocate proteins once they acquire tertiary structure. Signal peptides delay folding of these nascent pre-proteins and allow chaperone SecB to bind to the mature region of the pre-protein³². SecB stabilizes the unfolded state of these pre-proteins³⁴ and keeps them in a loosely folded state that is competent for translocation³⁵. This process can occur while the protein polypeptide chain is still being synthesized at the ribosome³⁶ or after synthesis is complete. This SecB-pre-protein complex binds to the ATPase protein SecA³⁷ which then binds to the translocon. Pre-proteins cross the membrane through the translocase (i.e., a complex formed between SecYEG and SecA) in a manner that is poorly understood. What is known is that the motor protein SecA drives translocation at the expense of the cellular energy currency Adenosine Triphosphate (ATP) as well as proton motive force (PMF). The final step of translocation usually involves signal peptidase cleavage of the signal peptides³⁸ thus allowing correct folding of mature protein in the periplasm³⁹⁻⁴¹.

Figure 1.2: Schematic illustration of Posttranslational translocation. Precursors (red) with cleavable signal sequence are synthesized at the ribosome. These precursors rapidly bind to the chaperone SecB which keeps them in an unfolded state competent for translocation. The SecB-precursor complex binds the SecA ATPase to form a SecA-SecB-precursor complex. This complex binds to SecYEG and uses ATP binding and hydrolysis to translocate the precursor proteins through the SecYEG protein conducting channel.

1.3.2. Bacteriorhodopsin: light driven proton pump of *Halobacterium salinarum*

Bacteriorhodopsin (BR) is one of the most extensively studied membrane proteins in general, as well as in the AFM community⁴²⁻⁴⁷. Hence this light driven proton pump was chosen as a model system to demonstrate that glass can be a viable substrate for atomic force microscopy of membrane proteins. When excited by green light ($\lambda = 500 - 650$ nm), BR generates a proton gradient by pumping H^+ across cell membranes and against an electrochemical potential. Cellular ATP synthases use this proton gradient to produce ATP, the energy currency of the cell.

BR molecules assemble into trimers. These trimers pack into a two-dimensional hexagonal lattice with 6.2 ± 0.2 nm intra-trimeric distance in the membrane, termed “purple” membrane for its color. The purple membrane is a specialized part of the cell membrane of *Halobacterium salinarum*, a salt-loving microorganism. Each BR molecule consists of seven transmembrane α -helices that surround a photoreactive chromophore retinal^{48,49}. Extracellular and cytoplasmic sides of purple membrane are distinct and can be identified by their inter-trimeric distance (Figure 1.3)⁴⁶. BR subunits assemble into trimers with inter-trimeric distance of ~ 2.8 nm on extracellular side while it is ~ 3.5 nm for the cytoplasmic side of the purple membrane.

Figure 1.3: High-resolution images of purple membrane⁴². **(a)** Purple membrane directly adsorbed onto a mica supporting surface. **(b)** Extracellular and **(c)** cytoplasmic surfaces showing the substructures and trimeric assembly of BR. Scale bars are 500 nm for panel a, 5 nm for panels b&c and 2 nm for b&c insets respectively.

1.3.3. Enzyme catalase

Enzymes are remarkable molecular devices that catalyze chemical reactions in biological systems⁵⁰. Enzymes are nature’s chemists that perform manifold chemical transformations needed for life. Enzymes are powerful, very specific, selective and able to display a very high activity. They lower the barriers to chemical transformations by

presenting molecular surfaces that selectively stabilize a transition state. Most of the known enzymes are proteins; although several catalytically active RNA molecules have also been discovered. For example, the Nobel Prize in Chemistry was awarded jointly to Sidney Altman and Thomas R. Cech in 1989 *"for their discovery of catalytic properties of RNA"*⁵¹.

Catalysis of chemical molecules takes place at the active site of the enzyme. Active sites capture chemicals by utilizing intermolecular forces. These chemicals are optimally oriented before making and breaking chemical bonds. Formation of wasteful by-products due to side reactions is usually rare in enzyme-catalyzed reactions. Chemical reactions are accelerated by enzymes by factors as much as a million or more. Rate enhancement for some representative enzymes is presented in Table 1.2.

Table 1.2: Rate enhancement of few enzymes⁵²

Enzyme	Nonenzymatic half-life	Uncatalyzed rate const (s ⁻¹)	Catalyzed rate const (s ⁻¹)	Rate enhancement
OMP decarboxylase	78,000,000 years	2.8×10^{-16}	39	1.4×10^{17}
Staphylococcal nuclease	130,000 years	1.7×10^{-13}	95	5.6×10^{14}
AMP nucleosidase	69,000 years	1.0×10^{-11}	60	6.0×10^{12}
Carboxypeptidase A	7.3 years	3.0×10^{-9}	578	1.9×10^{11}
Ketosteroid isomerase	7 weeks	1.7×10^{-7}	66,000	3.9×10^{11}
Triose phosphate isomerase	1.9 days	4.3×10^{-6}	4,300	1.0×10^9
Chorismate mutase	7.4 hours	2.6×10^{-5}	50	1.9×10^6
Carbonic anhydrase	5 seconds	1.3×10^{-1}	1×10^6	7.7×10^6

Catalases are protective enzymes that are present in virtually all aerobic organisms that are exposed to oxygen and many anaerobic organisms⁵³. They are responsible for catalyzing the breakdown of harmful hydrogen peroxide into water and oxygen molecules in two steps before it can damage the cellular components. First the heme of the enzyme is oxidized by hydrogen peroxide into an oxyferryl species and generates a porphyrin cation radical. Then a second molecule of hydrogen peroxide is used as a reductant of compound-I generating water, oxygen and the resting state of enzyme as follows⁵³.

Catalases have been studied for more than 100 years⁵⁴. They have been isolated and characterized from many different organisms and several crystal structures have been published, to name a few, bovine liver catalase (BLC)^{55,56}, *Penicillium vitale* catalase (PVC)^{57,58}, *Micrococcus lysodeikticus* catalase (MLC)⁵⁹, *Proteus mirabilis* catalase (PMC)⁶⁰, *Escherichia coli* catalase (HPH)^{61,62}, *Saccharomyces cerevisiae* catalase (CATA)^{63,64}, human erythrocytes catalase (HEC)^{65,66} and *Mycobacterium tuberculosis* catalase-peroxidase (KatG)⁶⁷ (see Fig 1.4 for crystal structure).

Figure 1.4: Crystal structure of *Mycobacterium tuberculosis* catalase-peroxidase⁶⁷.

1.4. Challenges

1.4.1. Glass as a substrate for AFM

Pioneering work from the Hansma lab has shown the first molecular resolution AFM images of BR on mica supports in 1990 (Fig 1.5a). However, they could not achieve molecular resolution on glass supports (Fig 1.5b)⁴⁵. Several groups have tried to achieve molecular resolution of protein on glass supports as it enables straightforward coupling of AFM to other single molecule techniques. Several groups have tried chemical treatments like salinization etc^{45,68,69}. But there is no simple protocol for glass similar to mica, the popular “gold standard” substrate in AFM community. If one could establish a straight forward protocol to treat glass and achieve molecular resolution of proteins, it

would be possible to couple well established single molecule techniques like TIRF, FRET, FIONA etc. to AFM.

Figure 1.5: AFM images of (a) purple membrane adsorbed to mica and (b) cationized ferritin bound to purple membrane on silanized glass⁴⁵. Image size $\sim 48 \times 48$ nm. It is clearly evident that panel *a* (mica support) shows molecular resolution.

1.4.2. Hovering over single molecular complex in near native conditions

Hovering of AFM tip over a gold nanobead attached to glass substrate in air has been well established⁷⁰. King *et al.* were able to hover the AFM for > 1 hr with < 5 pm drift/min. But that experiment was performed in air. When liquid is added to the system, complex noise entities involving hydrodynamics can become pronounced. Biological membrane samples involve lipids, which can themselves fluctuate in position both laterally and vertically. This adds another component of noise to such measurements. Hence, it would be a challenge to hover an AFM tip over a single macromolecular complex (such as a translocase) for long periods of time and quantitatively interpret the signal. In this thesis we take a first step in this process and evaluate the highly dynamic components of the system (SecA) in the absence of a membrane environment.

If one could successfully establish a technique to hover AFM tip over a single macromolecular complex for long periods of time in near native conditions and interpret the fluctuations that are observed, there are several biological questions that could be addressed. Below are outlined a few examples.

1.4.3. Translocation mechanism of Sec Translocon machinery

During translocation, SecA advances precursors through the SecYEG protein conducting channel but the mechanistic details of how this is achieved are unknown. What is known is that SecA utilizes energy derived from ATP hydrolysis. Real time investigation of this mechanical process via AFM is not possible if one could not stably hover the AFM tip over the translocon. Protocols to attach polypeptides to AFM tips have been well established⁷¹. As illustrated in Figure 1.6a, it is theoretically possible to study the translocation process by attaching a SecA-precursor complex to AFM tip and hover it over the translocon. Using this approach one could determine the step size (if discrete steps are indeed taken), the translocation rate, pausing, and stall force of the machinery. As illustrated in Figure 6b, it is also possible to study the interaction of a leader peptide with the translocon via hovering an AFM tip over the translocon.

Figure 1.6: Schematic illustration of possible ways to study (a) posttranslational translocation and (b) the interaction of leader peptide with Sec-translocon and its vicinity using US-AFM.

1.4.4. Catalytic activity of enzymes

Catalase can catalyze the breakdown of hydrogen peroxide into water and oxygen at the turnover rate of one million times per second. At each turnover, it releases enough heat to unfold itself. The mechanism by which it dissipates the heat so effectively is not known. One could potentially address this by hovering an AFM tip over the catalase molecule during turn over.

1.5. Approach

1.5.1. Atomic Force Microscopy

The atomic force microscope (AFM) was invented in 1986 by Gerd K. Binnig *et al.* at IBM research, Zurich⁵. Since its invention, AFM has drawn a lot of attention among other scanning probe microscopes (SPM) as a general tool for imaging, measuring and

manipulating matter at the nanometer length scale in the physics and biological community.

Historical perspective: scanning probe microscopy steadily evolved from the invention of the scanning tunneling microscope (STM) in the early 1980s. The two most prominent and versatile SPM instruments are the AFM and STM. Both are known for their simplicity and high (atomic) resolution. AFM, which does not require a conducting sample, couples the ability to apply and control forces at the sub-pN level to surfaces with sub-nanometer lateral precision⁷². Many research labs around the world are now equipped with SPMs that are quite popular in the study of matter at the nanoscale. SPMs work by positioning a sharp tip about a nanometer from the sample. Piezoelectric actuators allow the position of the tip to be controlled with a few picometers accuracy. The signal must be very sensitive to tip-sample separation in order to achieve high precision. With a sufficiently sharp tip and relatively flat sample, one can obtain a quantitative three dimensional surface topography with atomic resolution. However, resolution obtained on topographically complex objects such as proteins is typically ~1 nm.

STM can be operated in two well-known modes namely, constant height mode, i.e. the distance between tip and sample surface is held constant, and constant current mode, i.e. the tunneling current between the tip and sample surface is maintained constant.

Need for Atomic Force Microscope: An STM can quantitatively map the topography of the sample surface under study with atomic resolution, but the sample must be sufficiently conducting in order to support a sensitive tunneling current. This

limitation was realized in the early days of STM development. This motivated the development of AFM that does not rely on tunneling current, but depends rather on the inherent interaction forces between the sharp tip and the sample under study. This has led to many topographic studies of insulating materials (such as biological molecules) with high resolution.

AFM employs a sharp tip, with a typical radius of curvature in the single-digit nanometers. This tip is mounted on a cantilever, which is a few 10s to 100s of micrometers in dimension. When this tip is brought close to the proximity of a sample surface, the tip gets deflected due to forces acting between the tip and the sample surface. A laser beam is focused on to the cantilever to detect the deflection of the cantilever using a photosensitive diode. The cantilever can be moved in a raster fashion on the surface of the sample to get the topography of the surface (Figure 1.7).

Figure 1.7: Schematic of an Atomic force microscope.

AFM can be operated in many modes to acquire sample topography at the nanometer scale. Two well-known modes are contact mode and tapping mode. In contact mode, the force between the tip of the AFM and the surface of the sample can be calculated from the spring constant and the displacement of the cantilever on the basis of Hooke's law which states that

$$F = -kx \quad (4)$$

where x is the displacement of the cantilever from its equilibrium position along the surface normal, k is the force constant and F is the restoring force exerted by the cantilever. In tapping mode, the cantilever is driven near its resonant frequency. The amplitude, frequency and phase of the oscillation shift because of the interaction between the tip and the surface, and this in turn provides an indication of the forces involved. Contact mode operates in the repulsive region, whereas tapping mode operates in both the

attractive and repulsive regions (Fig 1.8). Depending on the type of the interaction between the tip and the surface, AFM can be used to characterize a number of physical properties of the material such as topography, friction, charge distribution, work function, local magnetic field, electronic spins and thermal conductivity.

Figure 1.8: A schematic plot showing change in tip-sample interaction forces as a tip approaches the sample surface. Attractive forces dominate when tip is approaching the surface, but when tip comes in “contact” with the sample surface, there are strong repulsive interactive forces. Contact mode operates in the repulsive region, whereas in tapping mode, the tip oscillates through both attractive and repulsive regions.

There are several ways to calibrate spring constant of AFM tips. To name a few⁷², thermal noise calibration, hydrodynamic damping calibration, calibration using a spring with known spring constant, added mass calibration and cantilever calibration benchmarks. A convenient and widely used method is to calibrate by measuring thermal noise⁷³.

The Hamiltonian of a harmonic oscillator that is fluctuating in response to thermal noise and is in equilibrium with its surroundings is given by

$$H = \frac{p^2}{2m} + \frac{1}{2} m\omega_o^2 x^2 \quad (5)$$

Where x is the displacement of the oscillator, p is its momentum, m is the oscillating mass, and ω_o is the resonant angular frequency of the system.

From the equipartition theorem,

$$\langle \frac{1}{2} m \omega_o^2 x^2 \rangle = \frac{1}{2} k_B T^2 \quad (6)$$

Where k_B is the Boltzmann's constant and T is the temperature.

and
$$\omega_o^2 = k/m \quad (7)$$

If one can measure the mean-square spring displacement, the spring constant can then be obtained as

$$k = k_B T / \langle x^2 \rangle \quad (8)$$

1.5.2. Ultra-Stable Atomic Force Microscopy

AFM has the capability to resolve individual atoms at room temperature in both air⁷⁴ and liquid⁷⁵. Being a mechanical device, it inevitably drifts in space over time. This instrumental drift in atomic force microscopy has limited imaging resolution, and has been a critical problem for decades. One method to overcome instrumental drift is to increase the scan speed⁷⁶⁻⁷⁹. But, during the course of imaging, often, it is impossible to precisely return to the same starting position, if one deliberately shifts the tip to a different location on the sample surface as it is not registered to the tip. To overcome come this limitation, King *et al.*, proposed using a laser based feedback scheme for tip stabilization⁷⁰. Apart from the traditional laser that is reflected from the top of the cantilever surface, two additional lasers were employed as illustrated in Figure 1.9. One can know the position of the tip and sample by detecting the back scattered light from the tip and silicon fiducial mark deposited on to the glass substrate⁸⁰ in three dimensions. The position of the tip is then adjusted to remain in a fixed position relative to the sample

frame of reference. Using this local sample frame of reference, the position of tip has been controlled in 3D with high precision in air (<40 pm, $\Delta f = 0.01 - 10$ Hz). With this atomic-scale registration of tip to the sample reference frame, one can envision experiments like monitoring of protein dynamics, protein translocation, enzyme catalysis etc. by hovering the tip over a protein for extended periods of time.

Figure 1.9: An artist's rendition of an optically stabilized Ultra Stable Atomic Force Microscope⁷⁰. Figure credit: Brad Baxley and Greg Kuebler, JILA, Colorado, USA.

2. Glass is a Viable Substrate for Precision Force

Microscopy of Membrane Proteins

2.1. Summary

Though ubiquitous in optical microscopy, glass has long been overlooked as a specimen supporting surface for high resolution atomic force microscopy (AFM) investigations due to its roughness. Using bacteriorhodopsin from *Halobacterium salinarum* and the translocon SecYEG from *Escherichia coli*, we demonstrate that faithful images of 2D crystalline and non-crystalline membrane proteins in lipid bilayers can be obtained on microscope cover glass following a straight-forward cleaning procedure. Direct comparison between AFM data obtained on glass and on mica substrates show no major differences in image fidelity. Repeated association of the ATPase SecA with the cytoplasmic protrusion of SecYEG demonstrates that the translocon remains competent for binding after tens of minutes of continuous AFM imaging. This opens the door for precision long-timescale investigations of the active translocase in near-native conditions and, more generally, for integration of high resolution biological AFM with many powerful optical techniques that require non-birefringent substrates.

2.2. Introduction

Atomic force microscopy has emerged as an important tool for macromolecular characterization in biological settings and is well suited for studying membrane proteins,

which are challenging to address using traditional techniques.⁸¹⁻⁸³ Employing a vanishingly sharp force probe affixed to a precise translation stage, an AFM is capable of imaging membrane proteins without resorting to freezing or crystallization. Operating in physiological salt solution without the addition of any labeling, AFM resolves protein protrusions above the lipid bilayer, revealing macromolecular structure and conformational dynamics in near-native conditions. Despite unique capabilities, AFM has yet to reach its full potential within the nanoscience research community due to its lack of seamless integration with advanced light microscopy methods.⁸⁴

Optical microscopy and spectroscopy tools are among the most broadly applied methods in biology. Common applications range from high throughput drug discovery assays based on fluorescence polarization⁸⁵ to fundamental biophysical studies utilizing super-resolution methods that routinely break the diffraction limit.^{8,86} Increasingly, optical microscopy techniques are being incorporated into AFM instruments to enhance functionality as well as precision.⁸⁷⁻⁹⁵ Local probe techniques are not able to resolve small molecules in solution. A combined AFM-single molecule fluorescence microscope⁹³ holds the potential to correlate ligand arrival with structural changes of a macromolecular target. Furthermore, AFM tips drift in space over time and experience forces in three dimensions. Inspired by techniques from the optical trapping microscopy community^{80,96,97} we have recently demonstrated an ultra-stable AFM⁸⁹ that minimizes positional drift as well as a means to directly observe three-dimensional tip-sample interactions.⁹⁴

High resolution (≈ 1 nm) biological AFM imaging^{82,98,99} has been carried out nearly exclusively using freshly cleaved mica as a specimen supporting surface, with a

handful of exceptions.^{69,100-102} This is due to freshly cleaved mica's inherent flatness, cleanliness, and biological compatibility. However, mica suffers from a fundamental limitation that has hindered its integration with numerous optical techniques. Mica exhibits biaxial birefringence; indeed, this naturally occurring material is used for optical wave plates. In general, propagation through birefringent material alters the polarization state and bifurcates the propagation direction of light in a manner which varies with material thickness. This makes it challenging to utilize freshly cleaved mica surfaces in modern optical systems, many of which employ highly focused and polarized laser beams passing through the specimen plane. Glass, on the other hand, is optically isotropic. It is a ubiquitous specimen supporting material for advanced optical microscopy methods.¹

In this work we sought to couple the benefits of glass substrates with high resolution biological AFM. To obtain an AFM image, membrane proteins are held to the supporting surface through a lipid bilayer, thus allowing studies in near-native environments. Ideally, the underlying surface should be chemically inert and timely to prepare. Thus we explored alternative approaches to silanization which have been reported in pioneering work.^{45,68,69} Using KOH-treated borosilicate glass cover slips as specimen supports, we demonstrate resolution of two integral membrane proteins at the level of monomer: bacteriorhodopsin, a bench mark sample in the field,⁴² as well as SecYEG, the bacterial translocon from *E. coli*. Additionally, we observe the association of the ATPase SecA with SecYEG, forming a translocase at the membrane interface. We suggest more generally that glass-supported lipid bilayers may be an effective mimic of the situation *in vivo* wherein numerous punctate contacts are made with membrane, for example, by cytoskeletal elements.¹⁰³

2.3. Results and Discussion

2.3.1. Glass treatment and reduction in surface roughness

As supplied by the manufacturer, borosilicate glass cover slips are rough on the molecular scale (Fig. 2.1a), exhibiting an average rms roughness of $19 \pm 9.6 \text{ \AA}$ (mean $\pm$ S.D., evaluated over $N = 100$ non-overlapping $100 \times 100 \text{ nm}^2$ areas). This limits their direct application in high resolution AFM. Treatment in saturated KOH ethanol solution reduces the roughness by approximately an order of magnitude (Figure 2.1b, roughness = $1.7 \pm 0.3 \text{ \AA}$, $N = 440$). We chose this approach because the etch rate of SiO_2 is known to plateau and then to decrease at high KOH concentrations;¹⁰⁴ acting as a moderator, alcohol simultaneously reduces the etch rate and increases the uniformity¹⁰⁵ of the etched surface (see Fig. 2.2 for alternative treatment methods). Though smoother, KOH-treated glass is still approximately 6-fold rougher than freshly cleaved mica (Fig 2.1e, roughness = $0.30 \pm 0.03 \text{ \AA}$, $N = 127$). The extremely flat nature of mica has advantages when carrying out imaging directly upon the solid-state surface, but our ultimate goal is to image membrane protein protrusions emanating from the upper leaflet of supported lipid bilayers.

Figure 2.1: Glass preparation and reduction of roughness. Comparison of untreated glass (a) with KOH treated glass (b) reveals over an order of magnitude reduction in rms roughness. A further roughness reduction was observed when KOH treated glass (c) was coated with lipid (d). In contrast, images of mica before (e) and after (f) lipid deposition show increasing surface roughness upon lipid coating. Average rms roughnesses are indicated in the bottom right of each panel. Panels a & b share the same 30 nm vertical color scale. Vertical scales for data (c-f) are identical (2 nm) and indicated. Line scan profiles (*white traces*) are shown through the center of the images. Scale bars for (a & b) are 200 nm; for panels (c-f) bars are 20 nm.

Figure 2.2: Glass treatment comparison. Panel a shows an image of a glass coverslip after immersion in hydrofluoric acid (HF; 48 wt. %, Sigma Aldrich) for ~ 3 seconds. Panel b is an image of glass after treatment in buffered oxide etchant (BD solution; Transene, Inc.) for ~ 3 min. Samples were rinsed in ethanol and distilled deionized water thoroughly prior to AFM investigation. Average rms roughness (evaluated in non-overlapping 100 X 100 nm² areas, $N \geq 100$) are indicated at the bottom right corner for each image. Data acquired in recording buffer (10mM HEPES pH 8.0, 200 mM KAc, 5mM MgAc₂) using MSNL tips (Bruker). Lateral scale bars are 200 nm, the vertical color scale for both images is identical and indicated. For comparison, both of these treatment methods yielded considerably rougher surfaces than saturated KOH ethanol solution, which produced an average rms roughness of 1.7 Å rms (see Fig. 2.1b & c and discussion in main manuscript). We also tested glass treatment in saturated KOH followed by HF, as well as saturated KOH followed by BD solution. In both of these cases the resulting glass surfaces were rougher than KOH alone.

Hence we explored the use of KOH-treated glass as a supporting surface for lipid bilayer imaging and compared results to those achieved with mica. Surprisingly, the difference in surface roughness between the upper bilayer leaflet imaged on KOH-treated glass and on mica is small (< 2 -fold; Fig. 2.1, compare panels **d** & **f**). This is noteworthy considering that untreated glass is approximately 60-fold rougher than mica itself. The effect comes about from two sources. First, the roughness of glass-supported samples is reduced, as can be seen when the same region is analyzed before and after deposition of *E. coli* polar lipid (Fig. 2.3). Sampling of 340 non-overlapping areas reveals the average rms roughness is diminished from $1.7 \pm 0.3 \text{ \AA}$ to $1.4 \pm 0.4 \text{ \AA}$ (Fig. 2.1c & **d**, respectively). We attribute the observed smoothing to the bilayer's ability to span local valleys in the complex topography of the glass surface. Second, in contrast to glass, the roughness of mica-supported samples increases upon lipid bilayer deposition to $1.0 \pm 0.2 \text{ \AA}$, $N = 365$ (Fig. 2.1, compare panels **e** & **f**). We attribute this roughening to lipid conformational fluctuations, which occur both laterally and vertically,¹⁰⁶ which also occur on glass, but which can only add disorder to the atomically-flat crystal plane of mica. Thus, for studying membrane protein protrusions, KOH-treated glass appears to be a suitable candidate for use as a supporting surface.

Figure 2.3: Direct visualization of the reduction of surface roughness via lipid deposition on glass. AFM images of the same area of KOH treated glass before (a) and after (b) lipid deposition. Line scan profiles are shown above the images. The change in rms roughness determined in approximately the same area (*white dashed boxes*) is shown. A fiducial mark (visible on the right side of both images, deposited on the cover slip via physical vapor deposition of amorphous silicon through a shadow mask⁸⁰) allows for image registration before and after lipid deposition. Data acquired in recording buffer (10mM HEPES pH 8.0, 200 mM KAc, 5mM MgAc₂) using biolever mini tips (BL-AC40TS, Olympus). Lateral scale bars are 200 nm, vertical color scales are identical and indicated.

2.3.2. Bacteriorhodopsin on glass

To substantiate this notion we imaged bacteriorhodopsin from *Halobacterium salinarum* deposited on KOH-treated glass cover slips and compared the data to that acquired on mica (Fig. 2.4). Bacteriorhodopsin forms a well characterized two-dimensional lattice which has become an effective resolution standard for the field.⁴² First, large scale AFM imaging was carried out to locate individual membrane patches, identified by their characteristic height (~ 5 nm) above the supporting glass surface (Fig. 2.4a). Smaller-scale imaging (Fig. 2.4b) revealed molecular resolution and periodicity

inherent in the lattice. Correlation averaged data (Fig. 2.4c, $N = 100$ iterations) was used to determine the ~ 3.5 nm inter-trimeric distance, which is characteristic of the cytoplasmic side of bacteriorhodopsin.¹⁰⁷ Resolution achieved depends on a number of factors and can vary with individual tips within the same lot (SNL-A, Veeco).^{42,107} Therefore, the same identical tip that had been used with glass was used to image the same side of bacteriorhodopsin supported by mica (Fig. 2.4e-h). Two dimensional Fourier transforms of both data sets exhibit peaks out to and slightly beyond a 1 nm^{-1} radius (Fig. 2.4d & h) indicating that similar resolution was achieved on glass as on mica. Therefore, using this benchmark membrane protein sample, we demonstrated that there is no major difference in image fidelity over the areas required to visualize individual bacteriorhodopsin monomers.

Figure 2.4: Molecular resolution imaging of bacteriorhodopsin on glass and comparison with mica. Large-scale image of purple membrane patch supported (a) by KOH-treated glass and (e) by mica. Smaller-scale imaging (b, on glass; f, on mica) reveals individual bacteriorhodopsin trimers. Correlation averaged and Fourier transformed data are shown (c & d, respectively, on glass; g & h, on mica). To facilitate direct comparison with glass substrates, data (e-h) was acquired using the same identical tip, but with a mica substrate. The asterisk in (c & g) indicates the center of the trimers. Scale bars are 200, 20 and 2 nm in (a & e), (b & f), and (c & g), respectively. The vertical color scales for a & e and b & f are 25 nm and 8 Å, respectively. The vertical color scale for c & g is 3 Å.

There is a small difference in trimer conformation between the two samples, which were imaged in different buffer conditions (*glass*: 20 mM Tris, pH ~ 8.5, 200 mM KCl, 20 mM MgCl₂; *mica*: 10 mM Tris, pH ~ 7.6, 150 mM KCl). The structure of bacteriorhodopsin depends strongly on the tip-sample interaction force as well as on the pH of the imaging buffer solution.^{108,109} When the pH of the imaging buffers was made

equal (pH ~ 8.5) the trimer conformations became more alike, although not identical (Fig. 2.5a and b, data acquired using a different tip from the same batch [SNL-A, Veeco] and different salt conditions). It is possible that differing interactions between the two solid supporting surfaces and the proteins account for residual differences in the observed bacteriorhodopsin conformations. However, standard deviation maps generated from the correlation averaging revealed a similar magnitude of conformational dynamics (Fig. 2.5c and d). This suggests that the underlying surface-protein interactions are not the primary cause of the conformational differences.

Figure 2.5: Bacteriorhodopsin conformation and conformational flexibility on glass and mica at pH 8.5. Bacteriorhodopsin trimers imaged at pH 8.5 on glass (a) and on mica (b). It is known that bacteriorhodopsin assumes different conformations at different pH values^{42,109,110}. Standard deviation maps (generated from correlation averages) on glass (c) and on mica (d) reveal a similar magnitude of vertical conformational flexibility (~1.5 Å), suggesting that the underlying surface-protein interactions are similar. The asterisk in (a & b) indicates the center of a trimer. Imaging buffer conditions were as follows, *glass*: 20 mM Tris, pH ~ 8.5, 200 mM KCl, 20 mM MgCl₂; *mica*: 20 mM Tris, pH ~ 8.5, 150 mM KCl. Data acquired using different tips from the same batch [SNL-A, Veeco]. Data in (a) and (b) was background subtracted and filtered (median, single pass). Lateral scale bars are 2 nm.

2.3.3. Visualization of SecYEG translocons in membrane

To explore the potential of glass beyond two-dimensional arrays of membrane proteins, we studied individual components of the general secretory system of *E. coli*. We have previously characterized this system on mica surfaces, relating structural observations in near-native conditions to biological function.^{111,112} Purified SecYEG translocons were reconstituted into liposomes and tested for translocation of precursor protein using established protocols.^{111,112} Active proteoliposomes were then deposited onto KOH-treated glass surfaces for imaging. Individual translocons, identified as punctate protrusions (Fig. 2.6a, c), were classified by their heights above the lipid bilayer. Following previous work,¹¹² cytoplasmic and periplasmic protrusions were identified by exploiting the asymmetry inherent in the SecYEG structure.¹¹³ The clear minimum in the height histogram at ~1.3 nm (Fig. 2.6b) separates the two orientations. The periplasmic orientation is indicated (Fig. 2.6b, *grey hatched*); cytoplasmic protrusions exhibit heights > 1.3 nm. In agreement with our previous study using mica substrates (Fig. 2.6b, *black dashed*, data from ref ¹¹²), there is a large distribution of heights for cytoplasmic SecYEG protrusions ranging from 1.3 to over 3 nm. This conformational diversity is likely to be due to dynamics of unstructured loops. There are two large (>30 amino acid) flexible loops connecting the ends of helices 6-7 and 8-9 of SecY.¹¹² Overall, these data indicate that the measured SecYEG protrusion topography is similar when imaged on glass and on mica.

Figure 2.6: Visualization of SecYEG translocons in membrane. **(a)** AFM image of a glass-supported lipid bilayer containing SecYEG. A cross section profile (*white trace*) is also shown. Panel **(b)** shows height histograms of SecYEG on glass (*red*, $N = 1203$) and on mica (*dashed black*, $N = 2766$). Data was normalized to the total features, the fraction of occurrences in each bin of width $1.7 \text{ \AA}$ was plotted, the narrowest distribution was taken as the reference and the most highly populated bin was set to 1. An individual SecYEG monomer imaged on glass is shown **(c)**. Scale bars are 100 nm, and 5 nm for panels **(a)** & **(c)**, respectively.

2.3.4. Direct visualization of SecYEG-SecA interactions in real time

The peripheral membrane protein SecA is known to cycle on and off the translocon at the membrane,¹¹¹ forming a SecYEG/SecA complex. To demonstrate that activities at membrane interfaces can be imaged using glass substrates, we prepared proteoliposomes by coassembly of SecYEG and SecA which results in a highly active form of SecYEG,¹¹¹ and tracked individual translocons for $>1800 \text{ s}$. The presence (or absence) of SecA engaged on the translocon can be determined by protrusion geometry (Fig. 2.7a).¹¹¹ During the observation period, a molecule of SecA bound the cytoplasmic face of SecYEG at 170 s (Fig. 2.7b), disassociated at 1190 s, and then re-associated at 1360 s, indicating that the translocon remains competent for SecA binding over more than 30 minutes of continuous imaging. Therefore, a local probe can track and directly visualize intricate protein-protein interactions occurring on glass-supported lipid bilayers for extended time periods.

Figure 2.7: Direct observation SecA association with SecYEG. **(a)** Histograms of the maximum height of individual SecA/SecYEG complexes on mica (black dashed; $N = 1088$; bin size $4 \text{ \AA}$) and on glass (red; $N = 502$; bin size $4 \text{ \AA}$) surfaces. The fraction of occurrences in each bin was plotted, the narrowest distribution was taken as the reference and the most highly populated bin was set to 1. The prominent peak at $\sim 4 \text{ nm}$ is attributed to the height of the active SecYEG/SecA translocase and agrees well for data acquired on both surfaces. The peak between 1.5 and 3.0 nm corresponds to the height of the cytoplasmic protrusion of SecYEG in the absence of SecA. Periplasmic SecYEG protrusions which are $\lesssim 1 \text{ nm}$ and do not bind SecA were excluded from analysis. **(b)** Tracking membrane activities for over 30 minutes reveals SecA association, disassociation, and re-association on glass-supported lipid bilayers. At $t=0 \text{ s}$ the cytoplasmic SecYEG protrusion is visualized in the membrane. 170 s later SecA binds, as indicated by the significant change in protrusion geometry. SecA dissociates at 1190 s . At 1360 s , SecA has re-associated with SecYEG. The scale bar is 10 nm .

2.4. Conclusions

Glass cover slips are among the most widely used specimen supporting surfaces and are an appealing non-birefringent specimen supporting surface for use in biological AFM. Their adoption would expand the promise of force microscope applications throughout nanoscale bioscience and biotechnology. However, glass is significantly rougher than mica. We show that after a straight-forward cleaning process followed by lipid deposition, the difference in roughness between the upper bilayer leaflet supported by glass and by mica is minor (< 2 -fold). Further, using two different integral membrane proteins, bacteriorhodopsin from *Halobacterium salinarum* and the translocon SecYEG from *Escherichia coli*, we demonstrate that glass cover slips can be used as effective substrates for AFM of membrane protein protrusions without introducing undue distortions or compromising resolution. Finally, direct visualization of SecA associating

with the translocon during >1800 s of observation demonstrates that glass-supported SecYEG remains in an active configuration as evidenced by its competency for binding this critical peripheral subunit.

Single molecule measurement techniques have produced powerful biophysical insights. A promising future direction in this field lies in the ability to bring complementary techniques to bear on a single biologically active macromolecular complex. Our work provides a path for incorporating advanced optical techniques into local probe studies in a timely manner, enabling, for example, precision measurements of membrane activities in near-native conditions.

See appendix for methods and data analysis.

3. Real time single molecule visualization of nucleotide dependent conformational changes in SecA-ATP hydrolysis

3.1. Summary

SecA is a critical protein in *E. coli* that mechanically couples ATP hydrolysis to protein translocation through the SecYEG translocon. As in most ATPases, this process is thought to be regulated by conformational dynamics of specific domains but it is difficult to obtain information that directly visualizes these conformational changes. It is unclear how ATP hydrolysis controls the large-scale intra-domain dynamics that drive protein transport of both periplasmic and outer membrane proteins through the cytoplasmic membrane. Here we use atomic force microscopy to explore the nucleotide dependent conformational space of SecA in real time in near native conditions in the presence of ATP and non-hydrolyzable analogues thereof. Our data suggest that the internal plasticity of the ATPase is coupled to ligand binding that controls the allosteric regulation of the protein binding domain and the carboxyl domain. We demonstrate reversible switching between at least two different conformations. The data also imply that ADP binding allosterically regulates the protein binding domain dynamics while SecA explores a much more diverse dynamic conformational space in the presence of its energy currency ATP.

3.2. Introduction

More than 30% of newly synthesized bacterial preproteins are exported across the cytoplasmic membrane to their final destinations where they acquire their native folded functional states¹¹⁴. Several sophisticated export mechanisms have evolved to serve this biogenesis, but only the general secretory system (Sec) pathway is ubiquitous in all domains of life¹¹⁵. Around 96% of the pre-proteins that are exported (i.e., the exportome) are translocated through the Sec pathway in *Escherichia coli*¹¹⁶. SecA, an ATPase enzyme, binds to SecYEG to form a translocase that mediates chemo-mechanical conversion to translocate pre-proteins across the cytoplasmic membrane.

Peripheral membrane protein SecA is a large multi-domain protein (901 aminoacyl residues, Figure 3.1)^{111,117}. It is known to exhibit remarkable dynamics and flexibility^{114,118,119}. For example, its domains are known to show remarkable conformational plasticity, i.e. move relative to one another (Figure 3.2). Several models have been proposed to explain protein translocation with different roles for SecA. Inter-domain allosteric regulation in SecA plays a vital role in all of them¹¹⁴. SecA acts as a monomeric processive motor in a ‘piston’ model where extended mature domains move along its clamp^{120,121}. The tip of two helix finger has been proposed to latch onto the preprotein chain and push it through the SecYEG pore¹²². The preprotein is then translocated through the channel via ATP hydrolysis-driven power strokes in a step wise fashion¹²³. In another model, SecA is proposed to control SecYEG pore opening and closing through ATP hydrolysis, hence acting as an ‘allosteric channel regulator’. This facilitates a Brownian ratchet mechanism where the preprotein undergoes proton motive

force driven diffusion through the SecYEG channel^{122,124,125} and SecA acts as a ‘brake’ to prevent backsliding of the preprotein^{123,126}.

Figure 3.1: Domains of SecA. Structure of protomer of SecA (PDB code: 2FSF) in ribbon representation (*panel a*) and surface representation (*panel b*) with PBD modelled in based on *Bacillus subtilis* (PDB code: 1TF5). Color codes (*panel c*): nucleotide binding domain 1, NBD1 (*red*); PBD (*pink*); nucleotide binding domain 2, NBD2 (*blue*); variable domain, VAR(*cyan*); linker helix (*yellow*); helical scaffold domain, HSD (*purple*); helical wing domain, HWD (*green*); two helix finger, IRA1 (*orange*); and carboxyl-terminal domain, CTD (*cyan*)¹¹¹.

Major insight into the biomolecular structure and conformational states of SecA has come from X-ray crystallographic studies. Several different conformational snapshots have been recorded for SecA^{21,127-130}. The N-terminal of SecA (residues 1 – 619) consist of three domains, namely, two nucleotide-binding folds (NBD1, *red* and NBD2, *blue*; Figure 3.1) and a Precursor Binding Domain (PBD, *Pink*; Figure 3.1). ATP binds at the interface of NBD1 and NBD2. The energy released during hydrolysis has been proposed to allosterically mobilize the PBD and the C domain (residues 620 – 901, start of Helical Scaffold Domain (HSD) to the C terminus). Five forms of SecA have been crystalized in

the presence of different ligands and have exhibited three major conformational states of SecA (Figure 3.2). It has thus been hypothesized that the PBD domain of SecA can swivel from wide-open to a closed state in a ligand dependent manner^{21,25,114,121}. An interesting and complementary line of inquiry to the static crystal structures would be to directly observe the dynamic motion of SecA during catalysis using single molecule methods in near native conditions.

Knowing the nature and timing of individual steps of the catalytic cycle and associated conformational changes of SecA during ATP hydrolysis holds the key to understanding the protein translocation reaction. How a large mechanoenzyme like SecA harnesses the energy released during ATP catalysis and translates it into functional conformational transitions to drive nascent protein chains through the translocon remains a fundamental question in the bacterial protein translocation process.

Figure 3.2: Conformational states of SecA^{21,25,114,121}. The wide open state obtained from *Bacillus subtilis* (left, PDB code: 1M6N), open state obtained from *Bacillus subtilis* (middle, PDB code: 1TF5) and closed state obtained from *Thermotoga maritima* (right, PDB code: 3DIN; ADP-BeF bound) are shown. The PBD and C-domains are represented in pink and brown correspondingly.

Here we employ atomic force microscopy¹³¹, known for its ability to image proteins in near native conditions without resorting to freezing or crystallization, to elucidate the allosteric regulation of nucleotide coupled conformational states of SecA. All the nucleotides studied (ATP, ADP and ADPAIF₃) induced conformational changes

to the unbound (apo) state of SecA. ADP binding at the nucleotide binding domains allosterically induces movement in the PBD. Additionally, ATP hydrolysis at the catalytic site regulates the PBD and C domain conformational state. Our data combined with previous studies suggest that ATP catalysis allosterically drives major conformational changes through exquisitely balanced large interdomain motions which, in turn, drive preprotein translocation.

3.3. Results and Discussion

Previously, our group studied the topography of SecA/SecYEG complexes in lipid bilayers^{111,119}. In order to gain further traction on this complex membrane-bound system, in this work we study the conformational dynamics of SecA alone. We demonstrate single Ångstrom-scale changes in protein topography that depend on particular domains (and deletions) as well as nucleotides. Accurately mapping these changes should enable us (and others) to quantitatively interpret the AFM measured topography of fully active translocases in future work.

Here, we studied the role of the PBD and carboxyl domains in SecA ATP hydrolysis in near native conditions by using atomic force microscopy. Proteins were adsorbed onto mica and topographies were characterized in various conditions. Height, volume and areas of each individual macromolecule accessible to the AFM tip were measured and collected into histograms for statistical analysis.

3.3.1. Single molecule studies of the wild type SecA ATPase

SecA ATPase (WT) was adsorbed onto mica in near native conditions and studied using atomic force microscopy. Figure 3.3 shows a representative image. A higher magnification image of the same is shown in the inset of Figure 3.3. A histogram

showing the distribution of maximum feature height for individual SecA molecules in the absence of any nucleotide is presented (Figure 3.4, *black*). The histogram shows that the majority of features exhibit a maximum height of around ~ 4 nm. A more detailed view shows a clear minimum separating two sub-populations that are ~ 5 Å apart, (which is well above ~ 1 Å vertical resolution of the technique¹³²). A third sub-population in the height distribution is observed as a shoulder in the distribution at ~ 5 nm (see Fig 3.6a for details). These maximum height distributions of SecA-WT are in good agreement with the previously reported maximum height distributions of the SecA-WT protrusions bound to SecYEG above the lipid membrane using the same technique^{111,119}.

Figure 3.3: AFM topography images of SecA-WT on mica. Inset shows three SecA molecules.

To study the SecA-ATP hydrolysis phenomenon, WT SecA macromolecules were exposed to $\sim 100\mu\text{M}$ ATP in solution for ~ 15 min and then imaged in near native conditions in the absence of ATP in the imaging buffer. The concentration of ATP chosen for this study is known to be many fold higher than the reported affinity ($k_d \sim 267$

nm)¹³³. The height distribution profile of these molecules is presented as a histogram in Figure 3.4, *red*. ATP exposure has clearly shifted the maximal height distribution of the SecA ATPase to lower heights by ~ 4 Å. Pelz *et al* reported similar subnanometer enzyme mechanics in adenylate kinase when exposed to ATP¹³⁴. They observed a partially closed conformational state with dominant energetic minimum that would allow rapid product-substrate exchange.

Key insights into the conformational states of protein macromolecules have come from X-ray crystallography. Structures of SecA bound to SecYEG, bound to ADP and in the apo state indicate the PBD domain undergoing large scale swivel motion as shown in Figure 3.2. It is important to note that in the X-ray structures of SecA from *E. coli* (PDB code: 2FSF), the PBD region is not fully resolved. This is likely an indication that the PBD populates many diverse conformations without having one state more stable than the others. Between the wide open and the SecYEG bound state (closed state, Figure 3.2), PBD undergoes a large rotation of around 80° ¹³⁵. The PBD makes contact with the C-terminal helices in a closed conformation in all available structures of SecA. It has been proposed that the wide open state to closed state transition occurs in two distinct phases. Initially the PBD and the C-domain move as a single unit and then the PBD moves alone in the second phase, towards NBD2 to the fully closed state¹³⁶. Hence, we provisionally attribute the shift observed in the maximum height distributions after ATP exposure (Figure 3.4) to a change in conformations of the PBD domain and/or C-domain (Figure 3.2).

Figure 3.4: ATP exposure changes the AFM-measured heights of SecA. Height histograms of SecA on mica before (*black*, $N \sim 3700$) and after (*Red*, $N \sim 3400$) ATP exposure.

To further localize the ATP dependent shift in the height distributions, a mutant was designed by deleting the PBD domain (SecA Δ PBD) and studies were conducted in different ATP conditions. Previous biochemical studies have shown that mutating the PBD domain does not affect the basal catalytic activity of SecA-ATPase¹³⁷.

3.3.2. Single molecule studies of SecA Δ PBD mutant

SecA Δ PBD mutant was adsorbed onto mica in near native conditions and studied using atomic force microscopy. Figure 3.5 shows a representative topographic image. High resolution images of the same are shown in the inset of Figure 5. Histograms of maximum height are presented in Figure 3.6b. The height population exhibits a dominant peak at ~ 4 nm. When compared to the WT species (Fig. 3.6a), a sub-population in the maximum height distribution at ~ 5.2 nm is not present for the mutant. Hence this ~ 5 nm shoulder in the SecA-WT height distribution can be attributed to the PBD domain.

Figure 3.5: AFM topography images of SecA Δ PBD mutant on mica. Inset shows high resolution AFM topographies two individual SecA mutants.

Figure 3.6: PBD mutation affects the height distributions. Height histograms of SecA on mica, before (*panel a*, black, $N \sim 3700$) and after (*panel b*, black, $N \sim 4000$) PBD deletion. Note that the pronounced shoulder at ~ 5.2 nm in the WT species (*orange fit*) disappears after the PBD deletion.

3.3.3. Single molecule studies in various ATP conditions

To further investigate ATP induced conformational dynamics of the SecA-ATPase, the wild type and SecA Δ PBD mutant were subjected to four different ATP assays on mica as follows:

- (i) SecA-WT/SecA Δ PBD mutant not exposed to ATP after protein purification
- (ii) SecA-WT/SecA Δ PBD mutant exposed to $\sim 100\mu\text{M}$ ATP in solution for ~ 15 min and then imaged in near native conditions in the absence of ATP in the imaging buffer
- (iii) SecA-WT/SecA Δ PBD mutant exposed to $\sim 100\mu\text{M}$ ATP in solution for ~ 15 min and then imaged in the presence of $\sim 100\mu\text{M}$ ATP in the imaging buffer (as ATP was consumed)
- (iv) SecA-WT/SecA Δ PBD mutant exposed to $\sim 100\mu\text{M}$ ATP in solution for ~ 4 hrs and then imaged in near native conditions in the absence of ATP in the imaging buffer

As a complement to maximum height analysis (shown in Figure 3.9), an areal footprint analysis of the protein molecules, i.e. the area the molecules projected onto the mica surface, was also measured in the above stated conditions. These areal foot print distributions are presented as histograms in Figure 3.7 (SecA-WT) and Figure 3.8 (SecA Δ PBD mutant). It is evident that in case of SecA-WT, when exposed to ATP, its areal footprint increases from $\sim 200\text{nm}^2$ to $\sim 300\text{nm}^2$ in all studied conditions (Fig 3.7: primary peak position: black, 200 nm^2 ; red, 280 nm^2 ; green, 300nm^2 ; and blue, 280 nm^2), an indication that the protein macromolecule is undergoing into a conformational state that is exploring much wider conformational space. Alternatively, this may be a consequence of volume conservation associated with an overall softening of the molecules upon ATP exposure but, in the case of the SecA Δ PBD mutant, the main peaks

of the areal footprint populations remain the same, even after ATP exposure (Fig. 3.8, first peak position: black, 230 nm²; red, 230 nm²; green, 230nm²; and blue, 235 nm²). This is an indication that the increase in areal footprint of SecA-WT after ATP exposure is, in fact, due to the PBD motion, and not the consequence of volume conservations associated with overall softening of the molecule upon ATP hydrolysis. Our observations are consistent with the PBD domain exploring a much wider conformational space when exposed to ATP. These results are consistent with previous NMR studies and biochemical studies^{118,138}, which indicated that ATP binding to SecA changes its conformational state. It has been proposed that ATP binding and hydrolysis leads to a shift in equilibrium between disorder and order of the nucleotide binding cleft and this shift is allosterically propagated to the PBD domain through the HSD¹³⁹ domain. Statistics of areal footprint distributions for WT-SecA and SecAΔPBD mutant in different ATP conditions are presented in table 3.1.

Figure 3.7: SecA-WT areal foot print increases when exposed to ATP. Areal foot print distributions of SecA ATPase in different ATP conditions: SecA ATPase not exposed to ATP after protein purification, black ($N \sim 3700$); SecA ATPase exposed to ATP in solution for 15 min and imaged in absence of ATP, red ($N \sim 3400$); SecA ATPase exposed to ATP in solution for 15 min and imaged in presence of ATP, green ($N \sim 2500$); and SecA ATPase exposed to ATP in solution for ~4hr and imaged in absence of ATP, blue ($N \sim 2500$).

Figure 3.8: The majority of the peaks of the SecA Δ PBD areal foot print distribution remain the same when exposed to ATP. Areal foot print distributions of SecA Δ PBD mutant in different ATP conditions: SecA Δ PBD mutant not exposed to ATP after protein purification, black ($N \sim 4000$); SecA Δ PBD mutant exposed to ATP in solution for 15 min and imaged in absence of ATP, red ($N \sim 1900$); SecA Δ PBD mutant exposed to ATP in solution for 15 min and imaged in presence of ATP, green ($N \sim 1500$); and SecA Δ PBD mutant exposed to ATP in solution for ~4hr and imaged in absence of ATP, blue ($N \sim 1100$).

Table 3.1: Statistics (mean areal footprint $\pm$ standard error of the mean) of the SecA-WT and mutant areal footprint distributions in different ATP conditions.

Assay	<i>N</i> - WT	SecA-WT	<i>N</i> - Δ PBD	Δ PBD
(i) apo	~3700	$480 \text{ nm}^2 \pm 6 \text{ nm}^2$	~4000	$380 \text{ nm}^2 \pm 4 \text{ nm}^2$
(ii) ATP – no ATP	~3400	$650 \text{ nm}^2 \pm 9 \text{ nm}^2$	~1900	$580 \text{ nm}^2 \pm 9 \text{ nm}^2$
(iii) ATP + ATP	~2500	$940 \text{ nm}^2 \pm 17 \text{ nm}^2$	~1500	$570 \text{ nm}^2 \pm 9 \text{ nm}^2$
(iv) 4hr	~2500	$450 \text{ nm}^2 \pm 6 \text{ nm}^2$	~1100	$330 \text{ nm}^2 \pm 8 \text{ nm}^2$

Figure 3.9 and 3.10 show maximum height distributions of SecA-WT and SecA Δ PBD mutant in different ATP conditions. The full width at half maximum (FWHM) and statistics are presented in Tables 3.2 and 3.3. The results indicate that SecA-WT is conformationally more dynamic as compared to the mutant. In particular, the FWHM of SecA-WT is larger than that of the mutant in all the four conditions studied (see Table 3.2). This difference can be directly attributed to the PBD domain and motions thereof. Conformational dynamics (as evidenced by an increase in the FWHM) are even more pronounced when the protein macro molecules are imaged in presence and absence of ATP (see Fig 3.11 for direct comparison; SecA-WT: red $\sim 21 \text{ \AA}$ and green $\sim 23 \text{ \AA}$ vs SecA Δ PBD: red $\sim 15 \text{ \AA}$ and green $\sim 15 \text{ \AA}$). In the case of the mutant, these traces exhibit a similar maximum height distribution, whereas in case of SecA-WT, these distributions shift to slightly higher heights when imaged in the presence of ATP. Apart from the PBD domain, the C domain is thought to be an additional regulator of SecA and ATP catalysis. Hence, the changes in maximal height distributions observed in the mutant can be attributed to the C domain¹¹⁸. In both cases, when the protein is exposed to ATP for 15 min and imaged after ~ 4 hrs delay, the protein appears to adopt a more uniform and

compact state (FWHM of WT ~ 16 Å, and mutant ~ 14 Å) with lower heights as compared to the other three conditions (Fig 3.9 & 3.10, compare blue traces to other traces).

Figure 3.9: SecA-WT maximum height distributions shift when exposed to ATP. Maximum height distributions of SecA-WT in different ATP conditions: SecA-WT not exposed to ATP after protein purification, dotted black ($N \sim 3700$); SecA-WT exposed to ATP in solution for ~ 15 min and then imaged in the absence of ATP, red ($N \sim 3400$); SecA-WT exposed to ATP in solution for ~ 15 min and then imaged in the presence of ATP, green ($N \sim 2500$); and SecA-WT exposed to ATP in solution for ~ 4 hr and imaged in the absence of ATP, blue ($N \sim 2500$).

Figure 3.10: SecA Δ PBD mutant maximum height distributions show lower FWHM as compared to SecA-WT when exposed to ATP. Maximum height distributions of SecA Δ PBD mutant in different ATP conditions: SecA Δ PBD mutant not exposed to ATP after protein purification, dotted black ($N \sim 4000$); SecA Δ PBD mutant exposed to ATP in solution for ~ 15 min and imaged in the absence of ATP, red ($N \sim 1900$); SecA Δ PBD mutant exposed to ATP in solution for ~ 15 min and imaged in the presence of ATP, green ($N \sim 1500$); and SecA Δ PBD mutant exposed to ATP in solution for ~ 4 hr and imaged in the absence of ATP, blue ($N \sim 1100$).

Figure 3.11: Comparison of ATP-induced shifts in the FWHM for WT and mutant SecA. (a) Maximum height distributions of SecA-WT exposed to ATP in solution for ~ 15 min and then imaged in the absence of ATP (red, $N \sim 3400$), and presence of ATP (green, $N \sim 2500$); (b) SecA Δ PBD mutant exposed to ATP in solution for ~ 15 min and then imaged in the absence of ATP (red, $N \sim 1900$), and presence of ATP (green, $N \sim 1500$).

Table 3.2: FWHM of the SecA-WT and mutant maximum height distributions in different ATP conditions

ATP Assay	N - WT	SecA-WT	N - Δ PBD	Δ PBD
(i) apo	~ 3700	~ 20 Å	~ 4000	~ 17 Å
(ii) ATP – no ATP	~ 3400	~ 21 Å	~ 1900	~ 15 Å
(iii) ATP + ATP	~ 2500	~ 23 Å	~ 1500	~ 15 Å
(iv) ~4 hr recovery	~ 2500	~ 16 Å	~ 1100	~ 14 Å

Table 3.3: Statistics (mean height $\pm$ standard error of the mean) of the SecA-WT and mutant maximum height distributions in different ATP conditions.

ATP Assay	N - WT	SecA-WT	N - Δ PBD	Δ PBD
(i) apo	~ 3700	44 Å $\pm$ 0.2 Å	~ 4000	42 Å $\pm$ 0.2 Å
(ii) ATP – no ATP	~ 3400	41 Å $\pm$ 0.2 Å	~ 1900	36 Å $\pm$ 0.2 Å
(iii) ATP + ATP	~ 2500	44 Å $\pm$ 0.3 Å	~ 1500	39 Å $\pm$ 0.3 Å
(iv) ~4hr	~ 2500	38 Å $\pm$ 0.2 Å	~ 1100	34 Å $\pm$ 0.3 Å

3.3.4. Single molecule studies in the presence of different ATP analogues

To further investigate the conformational changes we observed in the presence of ATP, both SecA-WT and the Δ PBD mutant were investigated in the presence of ADP as well as a nonhydrolyzable ATP analogue. ADP-AlF₃ is known to trap the ATPase in its transition state¹⁴⁰ and ADP bound SecA can be an effective representation of the initial state of the hydrolysis cycle as ADP needs to dissociate from SecA to allow another ATP to bind to begin the next cycle. Hence, investigating SecA in the presence of these

analogues would enable us to study these nucleotide stabilized conformational states or ‘locked states’ of the ATP cycle.

Figure 3.12: SecA-WT areal foot print when exposed to different ATP analogues. Areal foot print distributions of SecA-WT: not exposed to ATP after protein purification, black ($N \sim 3700$); exposed to ATP in solution for ~ 15 min, red ($N \sim 3400$); exposed to ADP in solution for ~ 15 min, green ($N \sim 6500$); and exposed to ADP- AlF_3 in solution, blue ($N \sim 4600$).

Areal foot print distributions are presented as histograms in Figure 3.12 (SecA-WT) and Figure 3.13 (SecA Δ PBD mutant). Statistics of areal footprint distributions with different ATP analogues for SecA-WT and mutant are presented in table 3.4. It is evident that in case of SecA-WT, when exposed to ATP or any of its analogs, its areal footprint increases when compared to the apo state (Fig. 3.12, first peak position: black, 200 nm^2 ; red, 280 nm^2 ; green, 220 nm^2 ; and blue, 235 nm^2). This suggests that the protein macro

molecule explores a much wider conformational space once it has been exposed to either ATP itself or the analogs tested here. In the case of other nucleotides (i.e. ADP and ADP- AlF_3), the change is minimal compared to ATP, but clearly above the signal-to-noise ratio of the analysis (see table 3.4). In case the of the SecA Δ PBD mutant, the population distribution remains essentially the same, even after exposure to ATP and its nonhydrolyzable analogues (Fig. 3.13, first peak position: black, 230 nm²; red, 230 nm²; green, 220nm²; and blue, 220 nm²). This is an indication that the increase in areal footprint of SecA-WT after exposing to ATP is due to the motion of PBD domain.

Figure 3.13: SecA Δ PBD mutant areal foot print when exposed to different ATP analogues. Areal foot print distributions of SecA Δ PBD mutant: not exposed to ATP after protein purification, black ($N \sim 4000$); exposed to ATP in solution for ~ 15 min, red ($N \sim 1900$); exposed to ADP in solution for ~ 15 min, green ($N \sim 3100$); and exposed to ADP- AlF_3 in solution, blue ($N \sim 2200$).

Table 3.4: Statistics (mean areal footprint $\pm$ standard error of the mean) of the SecA-WT and mutant areal footprint distributions with different ATP analogues.

Nucleotide	N-WT	SecA-WT	N- Δ PBD	Δ PBD
(i) apo	~3700	480 nm ² $\pm$ 6 nm ²	~4000	380 nm ² $\pm$ 4 nm ²
(ii) ATP	~3400	650 nm ² $\pm$ 9 nm ²	~1900	580 nm ² $\pm$ 9 nm ²
(iii) ADP	~6500	420 nm ² $\pm$ 4 nm ²	~3100	390 nm ² $\pm$ 5 nm ²
(iv) ADP-AlF ₃	~4600	360 nm ² $\pm$ 4 nm ²	~2200	320 nm ² $\pm$ 6 nm ²

Figure 3.14 and 3.15 represent maximum height distributions of SecA-WT ATPase and the SecA Δ PBD mutant in the presence of ATP and its non-hydrolyzable analogues. The FWHM of these data and associated statistics are presented in Tables 3.5 & 3.6, respectively. In the case of SecA-WT, the maximum height distribution clearly shifts towards lower heights when exposed to ATP and its analogues (Figure 3.14). We note that similar observations of height reductions in the presence of nucleotides has been reported previously for Eukaryotic cyclic nucleotide-modulated channels¹⁴¹. While in the presence of ATP, the maximal height distributions show two main peaks and a shoulder at ~ 5 nm, similar to SecA-WT that is not exposed to ATP (red & black traces; figure 3.14), the distributions in presence of the two non-hydrolyzable nucleotides are uniform (green & blue traces; Figure 3.14) and peak at slightly lower heights as compared to the apo-state (black trace; Figure 3.14). We propose that the majority populations observed in the cases of ADP and ADP-AlF₃ represent the nucleotide stabilized conformational states i.e. “locked states” of the corresponding nucleotides. Similar nucleotide stabilized conformational states have been reported in earlier studies for secA¹³³ and other protein macromolecules¹⁴²⁻¹⁴⁶ in biochemical and insilico studies.

In the case of the mutant, the maximum height distributions of the apo state & the ADP exposed state are similar (Fig. 3.15 black & green); additionally, the ATP & ADP- AlF_3 exposed states are similar (Fig 3.15, red & blue). Taken together with the wild-type data, these results clearly indicate that ADP binding induces a conformational state changes in the PBD domain (compare black & green traces in Figures 3.14 & 3.15, or see Figure 3.16 for a direct comparison). The maximum height distribution changes seen in the mutant in the presence of ATP and ADP- AlF_3 can be attributed to the motion of the C domain, which is another known additional regulator of SecA and ATP catalysis ¹¹⁸. Conformational dynamics (as evidenced by increases in the FWHM of height histograms) are more pronounced if the protein macro molecules are imaged in presence of ATP and ADP- AlF_3 (see Fig. 3.17 and Table 3.5 for direct comparison; SecA-WT: red ~ 21 Å and blue ~ 15 Å vs SecA Δ PBD: red ~ 15 Å and blue ~ 16 Å). In the case of the mutant, these traces have similar maximum height distributions and similar FWHM, while in case of SecA-WT, they are more distributed (i.e. FWHM is higher by ~ 6 Å) when imaged in presence of ATP. These differences can be attributed to the conformational dynamics of the PBD domain.

Figure 3.14: SecA-WT maximum height distributions when exposed to different ATP analogues. Maximum height distributions of SecA-WT: not exposed to ATP after protein purification, black ($N \sim 3700$); exposed to ATP in solution for ~ 15 min, red ($N \sim 3400$); exposed to ADP in solution for ~ 15 min, green ($N \sim 6500$); and exposed to ADP- AlF_3 in solution, blue ($N \sim 4600$).

Figure 3.15: SecA Δ PBD mutant maximum height distributions when exposed to different ATP analogues. Maximum height distributions of SecA Δ PBD mutant: not exposed to ATP after protein purification, black ($N \sim 4000$); exposed to ATP in solution for ~ 15 min, red ($N \sim 1900$); exposed to ADP in solution for ~ 15 min, green ($N \sim 3100$); and exposed to ADP- AlF_3 in solution, blue ($N \sim 2200$).

Figure 3.16: ADP binding allosterically regulates the PBD domain (a) Maximum height distributions of SecA-WT in the apo state, black ($N \sim 3700$) and exposed to ADP in solution for ~ 15 min, green ($N \sim 6500$); (b) Maximum height distributions of SecA Δ PBD mutant not exposed to nucleotide after protein purification, black ($N \sim 4000$) and exposed to ADP in solution for ~ 15 min, green ($N \sim 3100$)

Figure 3.17: Maximum height distributions of SecA-WT and mutant in presence of ATP and ADP-AlF₃ (a) Maximum height distributions of SecA-WT exposed to ATP in solution for 15 min, red ($N \sim 3400$) and exposed to ADP-AlF₃ in solution, blue ($N \sim 4600$). (b) Maximum height distributions of SecA Δ PBD mutant exposed to ATP in solution for 15 min, red ($N \sim 1900$) and exposed to ADP-AlF₃ in solution, blue ($N \sim 2200$).

Table 3.5: FWHM of the SecA-WT and mutant maximum height distributions exposed to different nucleotides.

Nucleotide	<i>N</i> - WT	SecA-WT	<i>N</i> - Δ PBD	Δ PBD
(i) apo	~ 3700	~ 20 Å	~ 4000	~ 17 Å
(ii) ATP	~ 3400	~ 21 Å	~ 1900	~ 15 Å
(iii) ADP	~ 6500	~ 18 Å	~ 3100	~ 17 Å
(iv) ADP-AlF ₃	~ 4600	~ 15 Å	~ 2200	~ 16 Å

Table 3.6: Statistics (mean of the height distribution $\pm$ standard error of the mean) of the SecA-WT and mutant maximum height distributions exposed to different nucleotides.

Nucleotide	<i>N</i> - WT	SecA-WT	<i>N</i> - Δ PBD	Δ PBD
(i) apo	~ 3700	44 Å $\pm$ 0.2 Å	~ 4000	42 Å $\pm$ 0.2 Å
(ii) ATP	~ 3400	41 Å $\pm$ 0.2 Å	~ 1900	36 Å $\pm$ 0.2 Å
(iii) ADP	~ 6500	42 Å $\pm$ 0.2 Å	~ 3100	42 Å $\pm$ 0.2 Å
(iv) ADP-AlF ₃	~ 4600	40 Å $\pm$ 0.2 Å	~ 2200	37 Å $\pm$ 0.3 Å

3.3.5. SecA ATPase inter-domain conformational dynamics at single molecule level

Imaging the SecA ATPase in the presence of non-hydrolysable ATP analogues indicated that the majority of the molecules are stalled in “locked” states but switching between ATP-bound and ADP-bound states is of fundamental importance for ATP hydrolysis. Hence, we wanted to further investigate if these stalled molecules can be driven out of stalled states to undergo reversible conformational changes. In fact, single molecule dynamic studies of enzymes undergoing catalysis are difficult and have been sparse and indirect, as molecules can remain inactive during AFM studies¹³². Here we

followed individual SecA ATPase molecules in real space and real time to directly visualize conformational dynamics associated with ATP hydrolysis.

Figure 3.18: SecA ATPase scanned in two dimensions and one dimension: High resolution two dimensional topographies of SecA-WT is shown in panel (a). Panel (b) shows a kymograph¹⁴⁷ of the protein of interest (dotted circle, panel (a)). Here the slow scan axis is disabled and the AFM tip scans in one dimension over the same area (neglecting drift). By generating protein ‘tubes’, kymographs enable us to observe conformational dynamics with much better temporal resolution (~ 100 ms in panel (b) vs ~ 52 s in panel (a)).

With traditional AFM techniques, temporal resolution is often limited by the bandwidth of the feedback electronics. Hence, in order to achieve the temporal resolution required to observe conformational dynamics of SecA-WT in the presence ATP, we scanned these protein macromolecules in one dimension rather than the traditional two dimensions (Fig 3.18). First a traditional two dimensional image was taken to identify the proteins of interest. Then the slow scan axis was disabled to repeatedly scan the protein of interest to monitor the ATP induced conformational dynamics in real time with high lateral and temporal resolution (~ 1 nm and ~ 100 ms) under physiological conditions. The resulting kymographs were achieved without the need for large fluorescent labels which can prevent free protein motion. We exploited the large difference in protein width

profiles in the presence and absence of ATP that was evident from the areal footprint distributions (e.g., Figure 3.7) to monitor the real time dynamics of the ATPase catalysis as the substrate was consumed.

Figure 3.19: Direct visualization of SecA-ATPase domain movements by AFM. (a) Kymograph showing conformational dynamics of SecA-ATPase in presence of ATP. (b) 3D representation of the same. (c) Height profiles of compact (*red*) and expanded states (*black*) for the line scans of the same color from panel a (dashed lines).

Figure 3.19 illustrates a kymograph, i.e. a one dimensional scan of SecA-WT as a function of time. Here, the protein exhibits at least two conformational states of different width profiles that switch back and forth (see line scans in Fig. 3.19c) and this apparent ‘flickering’ can be captured over extended periods illustrating the reversible nature of these ATP induced conformational changes in SecA. We hypothesize that this flickering process may represent a conformational change directly reflecting ATP binding and unbinding from the SecA-WT, but further work will be required to confirm this.

Figure 3.20a illustrates a kymograph of SecA-WT in the presence of ATP for more than ~75 s. Here the protein molecule flickers back and forth between a compact and a more open or splayed state. Figure 3.20b shows the maximum width distribution profile of this molecule for the same period of time. The reversible switching of the molecule between compact and splayed state is reflected in the histogram as two populations. A clear minimum at ~ 29 nm separates these two populations. The protein

appears to have favored the splayed state over compact state in this case. Specifically, it remains in the splayed state for ~84% of the time and 14% in compact state (Fig 3.20b, *red* and *green* curve fittings correspondingly).

Figure 3.20: Reversible conformational dynamics of an individual SecA-WT molecule. (a) Kymograph showing flickering behavior of SecA-WT in the presence of ATP for over 75s and (b) histogram of AFM-measured width ($n \sim 400$ scan lines) showing two majority populations (compact and expanded states). Note, width measurements are overestimates of the true molecular dimensions because the dimensions of the AFM tip (nominal radius ~ 8 nm) were not deconvolved from the analysis.

In conclusion, we studied enzyme dynamics during ATP hydrolysis of SecA. Our measurements, which were carried out in real time and real space via AFM, provided information into structural and dynamic information in near-native biological conditions. Moreover, the method required neither additional molecular labelling nor complex data interpretation. Our measurements provided direct evidence of nucleotide-dependent motion of the PBD domain. This domain is known to be in direct contact with precursor protein and hence plays a critical role in translocation. We further showed that SecA-ATPase molecules can undergo reversible conformational changes in the presence of ATP and that the protein molecules can be stalled in ‘locked states’ in the presence of non-hydrolyzable ATP analogues. We demonstrate here, a unique capability of AFM to study reversible conformational dynamics at single molecule level for the

characterization of ligand induced reversible conformational dynamics that would be difficult to investigate using any other technique, in near native biological conditions and in real time.

See appendix for methods and data analysis.

4. Catalase Enzyme Dynamics during Catalysis

4.1. Introduction

In 1894, Emil Fisher proposed the “lock and key” mechanism, i.e. substrates fit perfectly into active sites of the enzymes like a lock and key¹⁴⁸. Ever since, the catalytic activity of the enzymes has fascinated biochemists¹⁴⁹. For the past century, the focus has been mainly on interconversion of reactants to product molecules and the rate of catalysis, but explaining highly exothermic enzymatic reactions has been largely overlooked¹⁴⁹. Recently, Riedel *et al.* have investigated the origin of anomalous diffusion of several enzymes with high ΔH (change in enthalpy)¹⁵⁰. They attribute this anomalous diffusion to an acoustic wave generated by the heat released during enzyme catalysis.

Previous studies of the urease¹⁵¹ and catalase¹⁵² enzymes also showed enhanced molecular diffusion. These observations were interpreted as self-phoretic effects, i.e., diffusion generated by release of changed products from the surface of the enzyme¹⁵³. Riedel *et al.* alternatively propose that the heat released during the catalysis reaction by the enzyme could be responsible for this enhanced diffusion.

Using fluorescence correlation spectroscopy, Riedel *et al.* demonstrated that the diffusion of enzymes correlates with the rates of the reactions catalysed by the enzymes and the heat produced during the catalysis. They also reported that the anomalous increase in the diffusivity of the enzyme is proportional to the velocity of the reaction and the heat generated during the reaction. They also heated the catalytic center of the enzyme with a short laser pulse to simulate the proposed effect and observed qualitatively

the same anomalous diffusion observed during catalytic reactions. Hence, it seems that the heat generated during catalysis gives rise to the observed peculiar diffusion of the enzymes.

In order to explain the observed anomalous diffusion of enzymes, Riedel *et al.* proposed a model where the heat generated during each catalytic cycle is transmitted as a pressure wave through the enzyme creating a differential stress at the enzyme-solvent interface, which in turn propels the enzyme. The authors called it a ‘chemoacoustic’ effect. The mechanism by which the pressure wave propagates through the proteins remains uncertain.

Several alternative theories for ‘chemoacoustic’ effect have been proposed¹⁵⁴⁻¹⁵⁹. Golestanian proposed an alternative theory¹⁵⁷ for the enhanced diffusion that contradicts the theoretical model proposed by Riedel *et al.* In his model, the enhanced diffusion coefficient of the enzymes could be explained simply by a combination of global temperature increase in the sample container and enhanced conformational change of the enzymes during catalysis cycle.

We proposed experiments to directly observe enzyme catalase during catalysis. The ideal way to do this would be to image the enzyme during catalysis to look for any structural changes. An alternative approach would be to hover an AFM tip over the enzyme during catalysis. In this mode the AFM tip would not be in mechanical contact with the enzyme, but rather, within close enough range (~1 nm) to detect more nuanced effects. Specifically, the AFM tip could potentially be used as a “nanophone” to detect acoustic waves, if they are present as proposed in the ‘chemoacoustic’ model of enzyme propulsion.

The work described in this chapter is a necessary step towards performing a hovering experiment, with the tip of the ultrastable AFM held in a position clamp over an active enzyme. In particular, we established the conditions to achieve stable imaging of catalase adhered to a mica substrate. In so doing, we imaged several conformational changes of this highly active enzyme. Interestingly, we also observed that the number of catalase enzymes on the mica surface *increased* during the course of exposure to its substrate, suggestive of oligomeric state changes. These results provide guidance for an improved experimental design incorporating an enzyme with the potential to create a dimeric (or higher order) species with covalent crosslinking to prevent dissociation. These improved experimental conditions are met by the inorganic pyrophosphatase enzyme, which is the subject of current work in the laboratory.

4.2. Results and discussion

4.2.1. Single molecule studies of KatG-WT

Wild type Catalase (KatG WT) from *Mycobacterium tuberculosis* and mutated Catalase (KatG mutant C171A/C541A) have been studied on mica supports in aqueous buffer solution using atomic force microscopy and corresponding AFM images are shown in Figure 4.1 and 4.2. As evidenced in Figure 4.2 and corresponding analysis, mutating the two cysteines of the wild type enzyme at position 171 and 541 with alanines clearly affected the structural stability of the enzyme. It is evident from the height and volume histograms presented in Figure 4.3 and 4.4 that the mutation biases towards structures with smaller heights and volumes.

Figure 4.1: Representative AFM image of Wild type Catalase (KatG WT) from *Mycobacterium tuberculosis* on mica support in aqueous buffer solution. High resolution surface topographies of the same are shown in inset.

Figure 4.2: Representative AFM image of mutated Catalase (KatG mutant C171A/C541A) from *Mycobacterium tuberculosis* on mica support in aqueous buffer solution. High resolution topographies of the same are shown in inset.

Figure 4.3: Height histograms showing maximum heights of KatG WT features (*red*, $N \sim 1700$) and KatG mutant C171A/C541A (*blue*, $N \sim 3000$). Clearly, the mutant population is biased towards lower features compared to WT, but there is still a non-zero minority population in the mutant sample with heights around ~ 7 nm.

Figure 4.4: Histograms showing volumes of KatG WT features (*red*) and KatG C171A/C541A (*blue*).

4.2.2. KatG in presence of hydrogen peroxide

We designed experiments to study the effect of H_2O_2 activation on KatG-WT. Representative images are illustrated in Figure 4.5. A general result is that significant variations in heights were observed and these dynamics correlated with the presence of H_2O_2 . Further, H_2O_2 changed the overall topography of the proteins. Features > 4.5 nm seem to largely disappear in the presence of H_2O_2 , and the height of the smaller features also seemed to increase by ~ 4 Å as shown in Figure 4.6. Potassium iodide (KI) is known to quench H_2O_2 . Hence, KI was added to the protein assay and the ~ 4 Å increase in heights observed in presence of H_2O_2 disappeared and the height changes are reversed, indicating reversible conformational changes (see Figure 4.6). Similar conformational fluctuations have been reported earlier for other enzymes^{132,134,141,159-162}. Another general feature that we observed was that the presence of the substrate H_2O_2 increased the number density of enzymes found on the supporting surface (from ~ 200 per μm^2 to ~ 310 per μm^2 , see figure 4.5).

Figure 4.5: Representative AFM image of Wild type Catalase (KatG WT) from *Mycobacterium tuberculosis* on mica support in (a) aqueous buffer solution (b) aqueous buffer solution containing ~ 10 mM H_2O_2 to activate the enzymes and (c) aqueous buffer solution containing ~ 10 mM KI for quenching.

Figure 4.6: Height histograms of Wild type Catalase (KatG WT) from *Mycobacterium tuberculosis* on mica support in aqueous buffer solution (*black*), aqueous buffer solution containing ~10 mM H_2O_2 to activate the enzymes (*red*) and aqueous buffer solution containing ~10 mM KI (*blue*) for quenching.

4.2.3. Single molecule studies of KatG mutant

To further investigate the changes observed in the presence of H_2O_2 , KatG (C173A, C551A, C21S, S303C) mutant macromolecules (KatG mutant 2 from now on) were exposed to ~100mM H_2O_2 in solution for ~15 min and then imaged in near native conditions in the presence of ~30mM H_2O_2 in the imaging buffer. The height distribution profile of these molecules is presented as a histogram in Figure 4.7, *red*. The main peak in the height distribution at ~8nm that was present without H_2O_2 (Fig 4.7, *black*) largely disappeared after H_2O_2 exposure. We provisionally attribute this shift observed in the maximum height distribution after H_2O_2 exposure to a change in oligomeric state of the KatG molecules.

Figure 4.7: Height histograms of KatG mutant 2 on mica before (*black*, $N \sim 2200$) and after (*Red*, $N \sim 2300$) exposing to H_2O_2 .

Further assays were designed to investigate if it was the pre-imaging incubation in $\sim 100\text{mM } H_2O_2$ in solution or the presence of $\sim 30\text{ mM } H_2O_2$ in imaging buffer that lead to the vanishing of $\sim 8\text{nm}$ peaks in the height distribution. To study this phenomenon, KatG mutant 2 macromolecules were exposed to $\sim 100\text{mM } H_2O_2$ in solution for $\sim 15\text{ min}$ and then imaged in near native conditions in absence and presence of $\sim 30\text{mM } H_2O_2$ in the imaging buffer. The height distribution profile of these molecules is presented as a histogram in Figure 4.8. Clearly, exposing KatG mutant to H_2O_2 in solution reduces most of the $\sim 8\text{nm}$ population (Fig 4.8, *red*) and remaining small fraction of populations with heights $> 6\text{ nm}$ gets reduced when imaged in presence of $\sim 30\text{ mM } H_2O_2$. The FWHM of the distributions increases from $\sim 12\text{ \AA}$ (Fig 4.8, *red*) to $\sim 22\text{ \AA}$ (Fig 4.8, *green*) when imaged in presence of $\sim 30\text{ mM } H_2O_2$. Similar observations were made for the SecA protein in presence of ATP (see chapter 3, Fig 3.9).

Figure 4.8: Height histograms of KatG mutant 2 that was exposed to $\sim 100\text{mM}$ H_2O_2 in solution and imaged in absence (*red*, $N \sim 2700$) and presence of $\sim 30\text{mM}$ H_2O_2 (*green*, $N \sim 2300$).

Figure 4.9: Height histograms of KatG mutant on mica before (*black*, $N \sim 2200$) and after (*Red*, $N \sim 2300$) exposing to H_2O_2 . Red and purple boxes indicate ~ 1 nm bands around the majority peaks at ~ 3.6 nm and ~ 8 nm.

Figure 4.10: Volume histograms of KatG mutant on mica from ~1 nm bands from figure 9, before (*black*) and after (*Red*) exposing to H₂O₂. Red and purple boxes indicate ~1 nm bands around the prime peaks at ~3.6 nm and ~8 nm in the height histogram.

To further validate our assumption that the two majority peaks observed at ~3.6 nm and ~8 nm corresponds to different oligomeric states (see Fig. 4.7), we further deconvolved the peaks by taking volume histograms of the features present within a 1 nm bin width around the principle peaks (see Fig. 4.9 & 4.10). These bands correspond to ~42% (Fig. 4.9 & 4.10, *peak 1*), ~24% (Fig. 4.9 & 4.10, *peak 2*) and ~30% (Fig. 4.9 & 4.10, *peak 3*) of the corresponding height distributions. The volume of the features corresponding to heights from peak 3 (i.e., Fig. 4.9 & 4.10, *purple box*) are > 3X higher than that of the features corresponding to peaks 1 & 2 (i.e., Fig. 4.9 & 4.10, *red box*). This clearly indicates an oligomeric state change of KatG mutant 2 when exposed to H₂O₂. Similar observations of substrate induced oligomeric state changes have been reported earlier for other enzymes¹⁶³⁻¹⁶⁵.

4.2.4. Visualization of KatG mutant oligomeric state change at the single molecule level

We followed individual KatG mutant molecules in the presence of $\sim 30\text{mM}$ H_2O_2 in real space and real time to directly visualize conformations dynamics and oligomeric state changes during catalysis. Figure 4.11 illustrates individual KatG mutants tracked for ~ 510 s. During this observation period, at ~ 170 s, the volume and height of the feature (*dotted circle*) reduces to approximately half, consistent with an oligomeric state change. We take the small changes in height and volume observed from ~ 170 s to ~ 510 s to be indications of conformational dynamics. Similar observations of real time conformational dynamics and protein-protein interactions have been reported earlier using AFM.^{112,132,141,147}

Figure 4.11: Tracking KatG mutant dynamics for over ~ 510 s reveals KatG mutant 2 disassociation in presence of ~ 30 mM H_2O_2 . At $t=0$ s the KatG mutants protrusion is visualized on mica. ~ 170 s later katG mutant dissociates as indicated by the significant change in protrusion geometry (dotted circle).

4.2.5. Oligomeric state recovery in KatG mutants

To further investigate if the oligomeric state changes in KatG mutant are reversible, KatG mutant 2 macromolecules were exposed to $\sim 100\text{mM}$ H_2O_2 in solution and imaged after ~ 15 min delay in solution or $\sim 4\text{hr}$ delay in solution. Maximum height

and volume distributions of these assays are presented in Figure 4.12 and 4.13, respectively. In both cases, height distributions are similar and there are two major volume populations observed. Volumes of the two majority populations imaged after ~4hr delay in solution (Fig. 4.13, *black*) is ~2X that of the two major populations observed after ~15 min delay. This is suggestive of an oligomeric state change. The KatG mutant 2 appears to acquire a higher oligomeric state after all of the H_2O_2 is consumed and the oligomeric state changes are reversible.

Figure 4.12: Height histograms of KatG mutant 2 that was exposed to ~100mM H_2O_2 in solution and imaged in absence of H_2O_2 after ~15 min delay (*red*, $N\sim 2700$) and ~4hr delay (*black*, $N\sim 1800$).

Figure 4.13: Height histograms of KatG mutant 2 that was exposed to ~100mM H_2O_2 in solution and imaged in absence of H_2O_2 after ~15 min delay (*red*, $N \sim 2700$) and ~4hr delay (*black*, $N \sim 1800$).

4.2.6. Single molecule KatG mutant studies in various H_2O_2 conditions

To further investigate H_2O_2 induced conformational dynamics and oligomeric state changes, KatG mutant 2 macromolecules were subjected to four different H_2O_2 assays on mica as follows:

- (i) KatG mutant 2 not exposed to H_2O_2 after protein purification
- (ii) KatG mutant 2 exposed to ~100mM H_2O_2 in solution for ~15 min and then imaged in near native conditions in presence of 30mM H_2O_2 in the imaging buffer
- (iii) KatG mutant 2 exposed to ~100mM H_2O_2 in solution for ~15 min and then imaged in absence of H_2O_2 in the imaging buffer

(iv) KatG mutant 2 exposed to ~100mM H₂O₂ in solution for ~4hrs and then imaged in near native conditions in absence of H₂O₂ in the imaging buffer

Figure 4.14 illustrates the height vs volume plots of the above cases. Clearly, the KatG mutant 2 that is not exposed to H₂O₂ (panel **a**) exhibits a wide spread distribution lacking a preferential oligomeric state. KatG mutant 2 that was exposed to H₂O₂ in solution for ~15 min or ~4 hrs and imaged in absence of H₂O₂ (panel **c** & **d**) show two well separated populations that indicate different oligomeric states. KatG mutant 2 that was exposed to H₂O₂ in solution for ~15 min and imaged in presence of ~30mM H₂O₂ (panel **b**) exhibits a wide spread distribution indicating that the KatG mutants are highly dynamic and undergoing conformational changes when imaged in presence of H₂O₂.

Figure 4.14: Height vs Volume distributions of KatG mutant 2 in different H_2O_2 conditions: (a) KatG mutant not exposed to H_2O_2 after protein purification; (b) KatG mutant exposed to $\sim 100\text{mM}$ H_2O_2 in solution for ~ 15 min and imaged in presence of H_2O_2 ; (c) KatG mutant exposed to $\sim 100\text{mM}$ H_2O_2 in solution for ~ 15 min and imaged in absence of H_2O_2 ; and (d) KatG mutant exposed to $\sim 100\text{mM}$ H_2O_2 in solution for $\sim 4\text{hr}$ and imaged in absence of H_2O_2 .

In conclusion, the results presented here indicate that the KatG macromolecules undergo both conformational and oligomeric state changes in presence of H_2O_2 . Future experiments like hovering over the enzyme while it is undergoing catalysis are poised to shed light onto the proposed ‘chemoacoustic’ effect.

See appendix for methods and data analysis.

5. Conclusions and Future Directions

I have successfully established glass as a substrate to study both crystalline and non-crystalline membrane bound proteins using AFM. The results presented here broaden the available substrates for precision measurements using biological AFM beyond mica and open the door for combining powerful optical methods with scanning probe techniques. One can envision novel experiments using US-AFM⁷⁰ to capitalize on this advance. The proposed hovering experiments will help to shed light on questions involving mechanistic details of protein translocation, protein unfolding and enzyme catalysis that are of fundamental and longstanding interest to the biophysics community.

5.1. Protein translocation

From studies of the Sec-translocase, it is evident that high resolution images can be achieved and protein-protein interactions (e.g., association and dissociation events of SecYEG/SecA) can be visualized on both glass and mica supports using atomic force microscopy. Now with well-established protocols to image the SecYEG translocon alone and in complex with ATPase SecA, new questions related to these highly dynamic proteins of the general secretory pathway can be addressed. Using US-AFM, one can hover an AFM tip over the Sec-translocase to garner a novel real-time, real-space vista into this complex.

In the past decades, protocols have been well established by Dr. Randall's group to study the translocation of pre-protein chains into proteoliposomes and it is evident that SecYEG-SecA complex remains competent for translocation in a reconstituted

proteoliposome system.^{111,166} Hovering an AFM tip over a Sec-translocase would be futile if the membrane bound SecYEG-SecA complex deposited on glass substrate is not active. But if the membrane-bound complexes are competent for translocation, that would open doors for several single molecule experiments using US-AFM and other optical techniques coupled to the AFM. Hence, experiments were performed to test the translocation activity of SecYEG-SecA complex deposited on two substrates: glass and mica.

5.1.1. Translocation assay of membrane bound SecYEG-SecA complex on glass supports

Proteoliposomes are deposited onto glass and mica substrates and allowed to rupture to form continuous bilayers. Radiolabeled precursor proOmpA, SecB, and ATP generating system consisting of phosphocreatine and creatine phosphokinase are added to the system and incubated at 30° C for 8 min for the translocation to occur (Fig 5.1a). Proteinase K was added to digest the precursor that has not been translocated. Degraded and untranslocated precursor was removed by a cycle of rinsing. Figure 5.1b shows the radioactivity measurements. Glass exhibited enhanced activity of translocation (solid line, red) as compared to mica (dotted line, black) in the presence of ATP (Fig 5.1c). Activity in the absence of ATP is shown in violet as a control for the translocation activity. The average roughness of the underlying glass topography (~170pm, 100x100 nm²) is greater compared to that of mica (~30 pm, 100x100 nm²). Because lipid bilayers can span local valleys on glass topography (Fig. 5.1a) we posit that the higher translocation activity of precursor on glass is due to the fact that there is more space for it to move into as compared to mica.

Figure 5.1: Translocation assay of radio-labeled precursor. (a) Schematic illustration of translocation, (b) radioactivity measurements on glass with and without ATP, (c) plot showing intensity vs position across mica/glass support in violet/red box in panel b. activity of translocation in the presence of ATP on glass (*red*) and mica (*dotted black*). *Violet* line shows the activity in the absence of ATP as a control.

Now, one can envision attaching a precursor protein to an US-AFM tip using well established protocols¹⁶⁷ and follow its translocation across membrane as illustrated in Figure 5.2 and this type of experiment may be able to elucidate the mechanistic details associated with protein translocation at single molecule level such as the real-time translocation rate, pausing (if present), backsliding, etc.

Figure 5.2: Schematic illustrating protein translocation study.

5.2. BR pulling

From BR studies, it is evident that high resolution images can be achieved on glass supports using atomic force microscopy. Now with well-established protocols to image the BR, new questions related to protein unfolding pathways and energy landscapes can be addressed. Using US-AFM, one can unfold and refold the protein in a novel real-time, real-space manner (Fig. 5.3) and garner valuable knowledge into intermediate states of unfolding and refolding pathways with unprecedented positional precision¹⁶⁸.

Figure 5.3: Artistic illustration of protein unfolding experiment using AFM.

5.3. SecA conformational dynamics

From SecA ATP hydrolysis studies, it is evident that SecA is highly dynamic in the presence of ATP. Kymograph studies clearly indicate SecA flickering between compact and splayed conformational states with transitions on the ~ms time scale. These studies were done by scanning in one dimension. Scanning in one dimension rather than traditional two dimensions drastically improved temporal resolution of visualizing these conformational dynamics. One can use US-AFM to achieve even better temporal resolution (stop scanning altogether) by directly hovering an AFM tip over a fluctuating domain, as illustrated in Figure 5.4.

Figure 5.4: Illustration showing a hovering tip to study conformational dynamics of SecA.

5.4. Chemoacoustic effect

Figure 5.5: Illustration showing hovering of AFM tip to study the putative waves which have been predicted to occur during enzyme catalysis.

Single molecule studies of catalase enzymes have indicated that the enzyme is highly dynamic in presence of its substrate, H_2O_2 . Reversible conformational changes

and an increase in the FWHM of the height distributions have been observed in single molecule statistical studies. Using US-AFM one can envision an assay to directly observe enzyme catalase during catalysis as shown in Figure 5.5. An AFM tip can be potentially used as a “nanophone” to detect acoustic waves, to study the proposed ‘chemoacoustic’ model of enhanced enzyme diffusion.

APPENDIX

A. Glass is a viable substrate for Atomic Force Microscopy

Glass surface preparation: Glass coverslips purchased from Corning (18 × 18 mm, No. 1.5, catalog #: 2850-18) were used for the study. They were cleaned using KOH pellets (Sigma Aldrich, catalog #: P5958) dissolved in absolute ethanol (Fisher Scientific, catalog #: BP2818) as follows. Saturated KOH solution was prepared by mixing 90 g of KOH in 350 ml of absolute ethanol. This mixture was stirred using a magnetic stirrer in a 1L beaker until the solution turned dark orange in color (~4 hrs). Home built Teflon baskets were used to hold the glass cover slips along their periphery for treatment in the saturated KOH solution for 3 min while immersed in a sonicator (Branson 5510). Coverslips were then rinsed with deionized water (18.2 MΩ*cm) using a squirt bottle and transferred into a beaker to be sonicated in distilled deionized water for an additional 3 min twice, with rinsing in-between. Coverslips were then rinsed with 95% ethanol, dried using ultra high purity nitrogen gas, and stored in a desiccator. Over several days surfaces can lose their hydrophilicity.¹⁶⁹ Thus, immediately before use, surfaces were plasma cleaned to render them hydrophilic as described below.

AFM support design: Custom cut square coverslips (~ 13 X 13 mm) were attached to 12 mm diameter AFM specimen discs (TED PELLA, Product No. 16208) using epoxy (Devcon, part #: 20845). Care was taken to uniformly distribute the epoxy between the glass and specimen disc and to ensure it was devoid of air bubbles. Discs were left overnight for the glue to harden. Immediately prior to use, the support assemblies were plasma cleaned (Harrick Plasma PDC-001) in oxygen for 10 min at 250 mTorr using ~ 30

W forward RF power. The dimensions of glass coverslip were chosen to be slightly larger than that of the specimen disc, which minimizes exposure of the epoxy to the plasma as well as the imaging buffer solution.

SecYEG and SecA purification: The translocon, SecYEG, was purified from a strain C43(DE3) suitable for over expression of membrane protein¹⁷⁰ harboring a plasmid encoding *secY C329S, C385S, secE* with an N terminal His-tag, and *secG*.¹⁷¹ Cells were broken by passage through a French pressure cell (8,000psi), and the membranes were isolated by centrifugation and solubilized in dodecyl- β -maltoside (DBM). SecYEG was purified by chromatography, using a HisTrap column (GE Healthcare), and stored at -80°C in 20 mM Tris-Cl at pH 8, 0.3 M NaCl, 10% (wt/vol) glycerol, 0.6 mM DBM, and 2 mM DTT. SecA was purified as described,¹⁷² with the following modifications: intact washed cells were incubated on ice for 30 min with 8 mM EDTA to chelate Mg^{2+} in the cell envelope. The cells were pelleted and washed twice to remove the EDTA before being lysed by three cycles of freezing and thawing in the presence of lysozyme. The removal of EDTA before lysis is crucial to prevent the extraction of zinc from SecA. After centrifugation, SecA was purified from the supernatant by chromatography, using a QAE (TosoHaas) column. The purified protein was dialyzed into 10 mM Hepes at pH 7.6, 0.3 M potassium acetate (KAc), 2 mM DTT, and stored at -80°C . Concentrations of the proteins were determined spectrophotometrically at 280 nm, using coefficients of extinction as follows: SecA $78,900 \text{ M}^{-1} \cdot \text{cm}^{-1}$; and SecYEG, $45,590 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Proteoliposome preparation: Proteoliposomes were prepared as described elsewhere.^{111,112} Lipids (*E. coli* polar lipid extract, Avanti) in chloroform were blown dry

with N₂ and placed in a vacuum chamber overnight. A dry mechanical vacuum pump (XDS5, Edwards) was used to prevent backstreaming of oil, a potential contaminant. Dried lipids were suspended in 10 mM Hepes, pH 7.6, 30 mM KAc, 1 mM Mg(Ac)₂. Unilamellar liposomes were prepared by extrusion through membranes (~100 nm pore diameter, Liposofast, Avestin). To form proteoliposomes the liposomes were swelled, but not disrupted, using a ratio of detergent to lipids of 4.65 mM DBM to 5 mM lipids.¹⁷³ After swelling for 3 h at room temperature, the proteins to be incorporated were added: SecYEG at 5 μM, and for coassembly of SecA, SecA at 5 μM dimer. Incubation was continued for 1 h at room temperature followed by addition of BioBeads SM-2 (BioRad) to remove the detergent. The proteoliposomes were isolated by centrifugation at 436,000 x g, 20 min. at 4°C in a TL100.1 rotor (Beckman). The pellet was suspended in the same buffer and centrifuged again as above. The final pellet was suspended to give a concentration of approximately 8 mM lipid and 8 μM SecY. The suspension was stored at -80°C.

Bacteriorhodopsin preparation: Halobacterium salinarum strain S9 was grown and the purple membrane prepared as described.¹⁷⁴ The isolated purple membrane was suspended in distilled deionized water at 4.5 mg/ml bacteriorhodopsin. The concentration was determined using the extinction coefficient of the retinal chromophore at 568 nm ($6.3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and molecular weight 26,000 for the protein. This stock solution was stored at -20 °C.

AFM imaging: All AFM images were acquired in recording buffer at ~30°C in tapping mode using a commercial instrument (Asylum Research, Cypher). Care was taken to

control the magnitude of the tip sample force to ≈ 100 pN (estimated by comparing the free amplitude to the set point amplitude). Under such conditions, minimal protein distortion is expected.^{108,175} Spring constants were determined using the thermal noise method. Details for each sample preparation follow. ***Glass alone:*** The recording buffer was 10mM HEPES pH 8.0, 200 mM KAc, 5mM MgAc₂; the tip used for the data shown in Fig. 10a & b was MSNL (Bruker) with spring constant ~ 0.4 N/m, a biolever mini (BL-AC40TS, Olympus) was used for Fig. 10c-f with spring constant ~ 0.06 N/m. ***Bacteriorhodopsin on glass:*** A solution was prepared by diluting bacteriorhodopsin to 45 μ g/ml in 10 mM Tris, pH ~ 7.8 , 300 mM KCl buffer. Equal volumes of this solution and adsorption buffer (10 mM Tris, pH ~ 9.2 , 700 mM KCl) were mixed before depositing onto a freshly plasma cleaned glass support. After 1 hour incubation, the sample was rinsed with 10 volumes of recording buffer (20 mM Tris pH ~ 8.5 , 200 mM KCl, 20 mM MgCl₂). SNL (Veeco) tips with measured spring constant ~ 0.4 N/m were used. ***SecYEG and SecYEG/SecA complexes on glass:*** Proteoliposome stock solutions were diluted to 80 nM SecYEG, 80 μ M lipid in recording buffer (10mM HEPES pH 8.0, 200 mM KAc, 5mM MgAc₂), immediately deposited on a freshly plasma cleaned glass support and incubated for ~ 20 minutes, followed by rinsing with recording buffer. Biolever mini tips (BL-AC40TS, Olympus) with measured spring constants ~ 0.06 N/m were used. ***Bacteriorhodopsin on mica:*** Following established protocols,⁴² equal volumes of stock solution and recording buffer (10 mM Tris pH ~ 7.6 , 150 mM KCl) were mixed before depositing onto a freshly cleaved mica support. After a 1 hr incubation, the sample was rinsed with 10 volumes of recording buffer. SNL (Veeco) tips of measured spring constant ~ 0.4 N/m were used.

Variability in glass surfaces: Some glass cover slips exhibit defects and a sparse distribution of pits is a common defect mode. The presence of small holes in the underlying supporting surface does not deleteriously effect the majority of topographic determinations of membrane protein protrusions¹⁷⁶ (Fig. 17).

Figure A 1: Glass substrates can accommodate membrane protein imaging in the presence of defects. Glass cover slips can exhibit defects and a sparse distribution of pits is a common defect mode. Panels a and b show different glass coverlips treated with KOH solution as described in Methods. Though panel b exhibits a sparse distribution of pits it can still be used to extract useful data. White boxes in panels a and b indicate 100 X 100 nm² areas with rms roughness of 1.9 and 2.0 Å, respectively. Average rms roughnesses are listed at the bottom right of panel a and b and were calculated over 100 × 100 nm² non-overlapping areas, $N \geq 100$; their standard deviations are 0.2 and 0.5 Å, respectively. Panel c shows a surface similar to panel b after deposition of liposomes containing SecYEG. The inset shows an expanded view indicating that the presence of holes in the underlying supporting surface does not interfere with topographic determinations of many protein protrusions. The scale bar for panels a, b and c is 200 nm and for the inset of panel c is 50 nm. Data was acquired in recording buffer (10mM HEPES pH 8.0, 200 mM KAc, 5mM MgAc₂). MSNL (Bruker) tips were used for panels a & b, a biolever mini tip (BL-AC40TS, Olympus) was used for c.

AFM image analysis: As is typical, images were flattened ($\leq 2^{\text{nd}}$ order) to minimize background. To allow direct comparison of average root mean square (rms) roughness, all roughness calculations were carried out on 100 × 100 nm² non-overlapping areas with the same pixel density (1.9 nm/pixel). Individual protein protrusions were cropped using custom software (Igor Pro, WaveMetrics) and a flood mask of ~ 2 Å above the lipid bilayer was applied to isolate protein protrusions. Software then extracted

topographical data of individual protein protrusions above the bilayer. For the data shown in Fig. 15c and Fig. 16 we implemented tip deconvolution.^{177,178} The program used blind tip estimation to determine the bluntest tip that could resolve the image. The generated tip geometry was then removed from the image, outputting a deconvolved image that more closely approximated the sample topography. Correlation averages ($N = 100$ iterations, Fig. 13c; $N = 200$ iterations, Fig. 13g) and standard deviation maps (Fig. 14c & d) from the correlation averages were generated using SPIP software (Image Metrology).

B. SecA ATP Hydrolysis

Atomic force microscopy: All AFM images were acquired in recording buffer at $\sim 30^\circ\text{C}$ in tapping mode using a commercial instrument (Asylum Research, Cypher). Care was taken to control the magnitude of the tip sample force to $\lesssim 100$ pN (estimated by comparing the free amplitude to the set point amplitude). Under such conditions, minimal protein distortion is expected^{179,180}. Spring constants were determined using the thermal noise method. Biolever mini tips (BL-AC40TS, Olympus) with measured spring constants ~ 0.06 N/m were used. In the presented experimental studies, protein was diluted to 20 nM concentration in incubation buffer. Two different incubation buffer conditions were used. No substrate (apo) conditions: 10mM HEPES pH 7.6, 100 mM KAc, 5mM MgAc₂ ; saturating nucleotide conditions: 10mM HEPES pH 7.6, 100 mM KAc, 5mM MgAc₂, 100 μ M nucleotide. Protein was incubated in solution for ~ 15 min in solution to expose it to the corresponding nucleotide. A total of $\sim 150\mu\text{L}$ of this solution was deposited onto a freshly cleaved mica surface. After ~ 10 min incubation on mica, sample was gently rinsed ~ 10 times with the imaging buffer. Proteins were imaged either

in 10mM HEPES pH 7.6, 100 mM KAc or in 10mM HEPES pH 7.6, 100 mM KAc, 100 μ M ATP as specified.

Data Analysis

Images were 1st order flattened to minimize mica background. Individual protein protrusions were cropped using laboratory made routines in Igor pro (wave metrics) and heights and areas of each molecule were calculated. For kymograph width distribution histograms (figure 17), molecule width traces were analyzed by hand to avoid misinterpretation of values due to noise. Magic plot software was used to do curve fitting.

C. Catalase enzyme dynamics

Atomic force microscopy: All AFM images were acquired in recording buffer at ~30°C in tapping mode using a commercial instrument (Asylum Research, Cypher). Care was taken to control the magnitude of the tip sample force to $\approx$ 100 pN (estimated by comparing the free amplitude to the set point amplitude). Under such conditions, minimal protein distortion is expected^{179,180}. Spring constants were determined using the thermal noise method. Biolever mini tips (BL-AC40TS, Olympus) with measured spring constants $\sim$ 0.06 N/m were used. In the presented experimental studies, protein was diluted to 290 nM concentration in incubation buffer. Two different incubation buffer conditions were used. No substrate (apo) conditions: 40mM HEPES pH 7.6, 1mM NiCl₂; substrate conditions: 40mM HEPES pH 7.6, 1mM NiCl₂, 10 mM/30mM/100mM H₂O₂ nucleotide. Protein was incubated in solution for ~15 min in solution to expose it to the H₂O₂. A total of ~150 μ L of this solution was deposited onto a freshly cleaved mica

surface. After ~ 10 min incubation on mica, sample was gently rinsed ~10 times with the specified imaging buffer.

Data Analysis

Images were 1st order flattened to minimize mica background. Individual protein protrusions were cropped using laboratory made routines in Igor pro (wave metrics) and heights, volumes and areas of each molecule were calculated.

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