Intermediate Trapping Using Force Spectroscopy

Satavisa Jana

A dissertation submitted for the partial fulfilment of the BS-MS dual degree in Science

Indian Education of Science Education and Research, Mohali

April, 2017

Certificate of Examination

This is to certify that the dissertation titled "Trapping Intermediates Using Force Spectroscopy" submitted by Ms. Satavisa Jana (Reg. No. MS12069) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee, duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Arijit Kumar De Dr. Ujjal K. Gautam Dr. Sabyasachi Rakshit

(Supervisor)

Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sabyasachi Rakshit at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Satavisa Jana

Dated: April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sabyasachi Rakshit

(Supervisor)

Dated: April 21, 2017

Acknowledgement

I take this opportunity to thank all those without whose valuable contribution this work would not have materialised. First of all, my heartiest thanks to my supervisor, Dr. Sabyasachi Rakshit, for providing me this chance to work on such an exciting project. His valuable insights and suggestions were most useful in steering this project ahead.

I also thank the other committee members for their views and thoughts on my project.

I am very thankful to all my fellow lab members, Surbhi, Gayathri, Jagadish, Jesse, Deb, Nisha, Sai, Anuj, Naimat and Ankush for creating a very vibrant lab atmosphere. Their help has proven to be an invaluable asset during the entire project.

Also, a special thanks to my senior, Shwetha, who helped initialise this project. Her guidance, not only during this project, but all through out these five years at IISER, has been the most helpful.

Next, I would like to thank all the faculty members of the Department of Chemical Sciences here at IISER, Mohali. My learning experience would not have been so interesting and nurturing without them. Thanks to Prof. K. S. Viswanathan for being an inspiration and a guide to me all these years. His courses were something I enjoyed thoroughly and looked forward to eagerly.

Thanks to all my friends at IISER, specially Anishya, Mugdha, Vaishnavi and Anuj. All of them made the five years here the most memorable ones.

I would like to thank the Department of Science and Technology, MHRD, Government of India for providing me with the financial support in the form of the INSPIRE fellowship. I would also like to thank IISER, Mohali for providing me the infrastructure and facilities, like the library, the computer centre, the central analytical facility, among others that were needed to bring this project into fruition.

My gratitude to Dr. Mariano Carrión-Vázquez and Dr. Andrés M. Vera from the Instituto Cajal, Spain for providing me with a few of the DNA constructs for my project.

Lastly, I would like to convey my thanks to my parents and my sister for always being there for me. Their love, support and encouragement have been the constants in my life.

Satavisa

List of Figures

List of Tables

Notations

Abbreviations	**Full Form**
SrtA	Sortase A
AFM	Atomic Force Microscope
TIRF	Total Internal Reflection Fluorescence
PolyG	Poly Glycine
PEG	Poly Ethylene Glycol
SVA	Succinimidyl Valeric Acid
IMAC	Immobilised Metal Affinity Chromatography
SEC	Size Exclusion Chromatography
APTES	(3-Aminopropyl)triethoxy Silane
Srttag	LPETG amino acid sequence
PCR	Polymerase Chain Reaction
m-PEG-SVA	Methoxy-Poly Ethylene Glycol-
Cy3	Cyanine 3
HPLC	High Performance Liquid Chromatography
ESI-MS	Electrospray Ionisation Mass Spectrometry
FRET	Förster Resonance Energy Transfer
DESI	Desorption Electrospray Ionisation
ADR	Adverse Drug Reaction
IADR	Idiosyncratic type Adverse Drug Reactions
LC-MS	Liquid Chromatography Mass Spectrometry
TCSPC	Time Correlated Single Photon Counting

Contents

Abstract

Trapping of reaction intermediates has been used as an approach to study the mechanism of various reactions. It serves the purpose of providing a convincing proof as to whether a particular reaction is going via a certain pathway. Here we trap and study an intermediate that is formed during the enzymatic reaction of Sortase A using the help of Force Spectroscopy via Atomic Force Microscopy (AFM). We estimate the lifetime and the off rates of the formed intermediate.

Chapter 1

Introduction

Transpeptidases are a class of enzymes that covalently link two different proteins via a peptide bond. SortaseA is an enzyme that belongs to this class. It is a bacterial transpeptidase found in the Gram positive bacteria *Staphylococcus aureus*. *S. aureus* is a common pathogen that causes a wide range of diseases in human beings, starting from superficial skin lesions to life-threatening endocarditis. They are known to colonize 20% of the healthy person permanently and up to 50% transiently.[1] In the recent years, *S. aureus* has become a matter of concern due to the emergence of strains like the methicillin resistant *S. aureus* (MRSA) strains. This particular strain of the bacteria is antibiotic-resistant. The toxicity of such pathogens, along with our limitation to cure these diseases has resulted in increased mortality rates as well as usage of additional antibiotics and costlier treatments. Also, in today's world of globalisation, the risk of spreading of this strain is much higher in a very short period of time. Therefore, there is a need to develop antibiotics which have lesser probability of giving rise to resistant strains.

The pathogenicity of *S. aureus* is, to a major extent dependent on its adhesion-associated proteins and enzymes system.[2] These help the bacteria to adhere to the host cells prior to infecting the host. For many Gram-positive bacteria sortase enzyme covalently attaches these proteins and enzymes to the peptidoglycan of the cell wall (**Figure 1.1**)

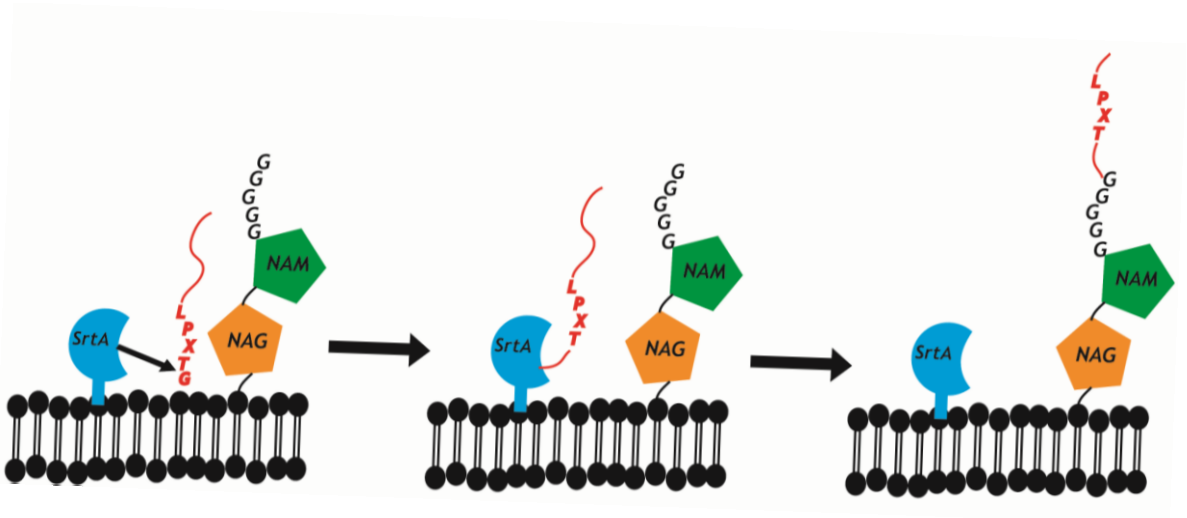

Figure 1.1: Schematic showing the SrtA reaction on the cell wall of the *S. aureus*. The SrtA recognises and attacks the LPXTG peptide sequence, forming the thio-ester intermediate. This intermediate is then attacked by the nucleophile, the pentaglycine sequence on the peptidoglycan of the cell wall, resolving the thio-ester intermediate.

The present day antibiotics mostly function by selectively blocking certain bacterial metabolic pathways. This ultimately results in the killing of the bacteria. But due to unregulated overuse of these antibiotics, microbial strains that are resistant to the antibiotics evolve over time due to the selection process. This causes the pharmaceutical industry to keep formulating newer antibiotics. By developing new antibiotics that target virulence strategies, like adherence to the host instead of the primary metabolic pathways of the pathogen, it can be assumed that the probability of emergence of a resistant strain to the particular antibiotic goes down.[3] This is because targeting the different virulence strategies does not affect the pathogen adversely, just disables it to infect the host.

The SrtA enzyme, as explained previously, plays an important role to adhere the pathogen to the host cell. Therefore, this enzyme can potentially serve as a future target for development of antibiotics.. Studying the enzymatic pathway of this enzyme in details could provide us with the necessary foundation required to further the search for a novel antibiotic.

SrtA is also a very important tool in biochemistry, biophysics and molecular biology. By the process of Sortagging,[4] a chemo-selective ligation technique, macromolecules can be modified with fluorophore, synthetic peptides and other functional units. This results in site-specific labelling of proteins. The target protein is engineered to have a sortase recognition site, a LPXTG tag. Sortagging has also been used to ligate lipid modified oligoglycine peptides to enhanced GFPs to form lipoproteins.[4]

SrtA mediated transpeptidation can also be used for fusion of two proteins or domains. Often for *in vitro* studies recombinant proteins need to be fused for functional characterization, etc. Using approaches like genetic fusions, though used widely, often lead to low yields of the unnatural product and misfolded proteins. In contrast, sortagging, being a post-translational method, allow native expression of the individual proteins that are then covalently linked.[4]

Proteins can also be immobilised on artificial surfaces using SrtA. This is an important use for surface fabrication for studies like SPR,[5] dynamic force spectroscopy,[4] etc. Site specific immobilisation of proteins can also be used for purification and separation of proteins.

The reaction mechanism of Transpeptidation by SrtA has been studied. It has been found that the reaction goes via a path of thioesterification followed by the transpeptidation step.[2] (**Figure 1.2**)

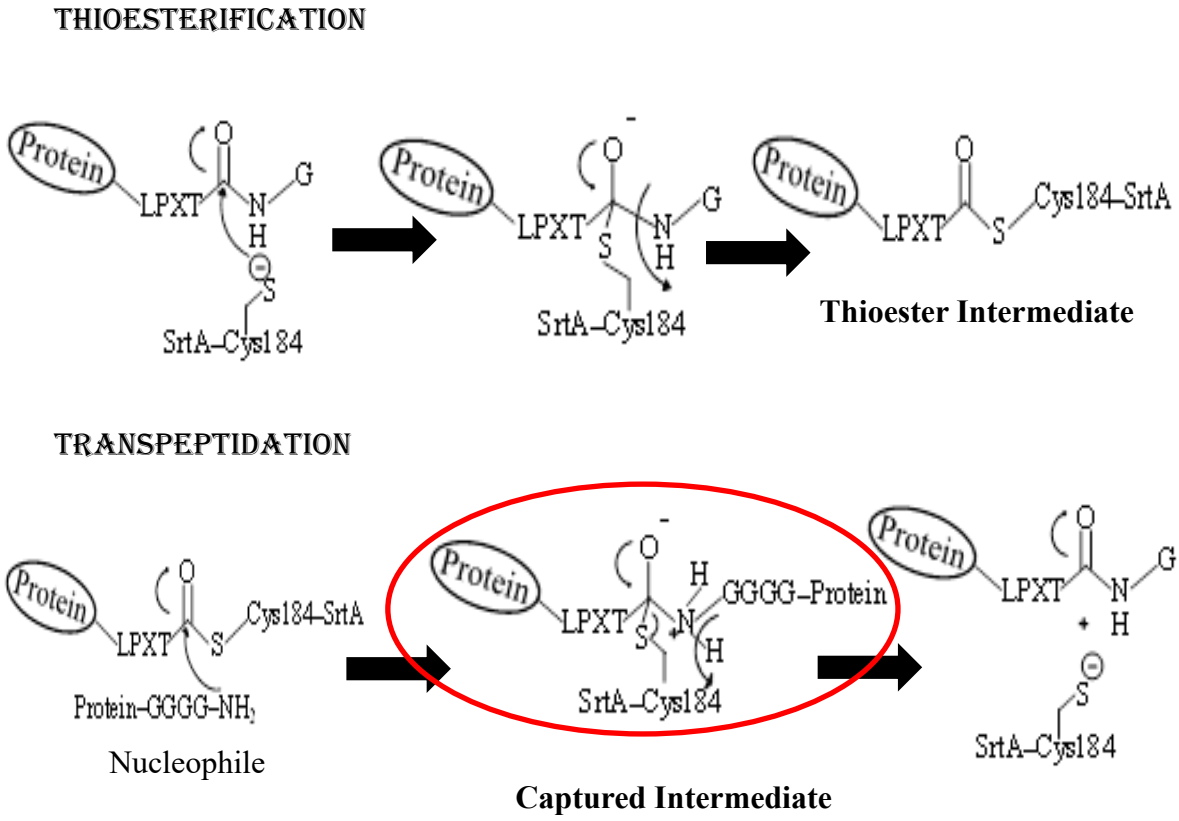

Figure 1.2: Schematic showing the enzymatic reaction mechanism of SrtA. The reaction begins with the LPXTG recognition by the enzyme, followed by formation of the thio-ester intermediate. Then there is a nucleophilic attack by the N-terminus of the pentaglycine motif which then ultimately leads to the formation of a new peptide bond between two proteins.

The recognition site for SrtA is a 5 peptide sequence: LPXTG. The C184 residue, in the thiolate form, attacks the peptide bond between the T and G amino acid forming the thioester intermediate. The formation of the thioester intermediate was shown by using hydroxylamine (NH_2OH).[6] Hydroxyaminolysis of acyl-enzyme intermediates give rise to hydroxamate products. In the mentioned study the detection of a threonine hydroxamate product confirmed the formation of thioester intermediate. This thioester intermediate is difficult to trap in the absence of any nucleophile since it undergoes hydrolysis. But in the presence of a nucleophile like polyG the thioester bond breaks and the N-terminus of the glycine residue is attached to the C-terminus of the LPXT amino acid sequence.

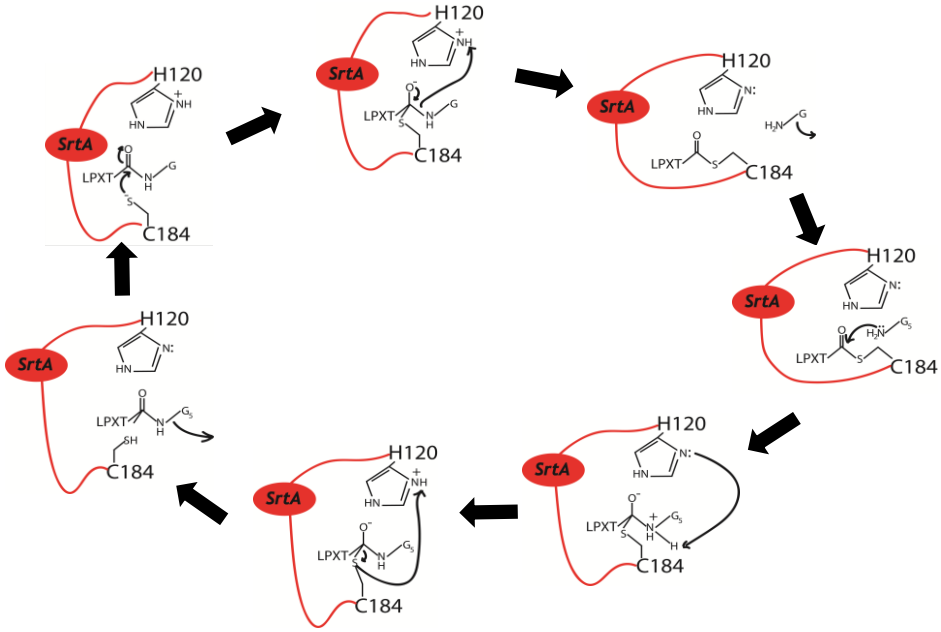

Figure 1.3: Schematic showing the enzymatic reaction mechanism of SrtA. Here the role of the H120, another amino acid of the enzyme has been depicted.

The other amino acids that are present in the active site of the enzyme play key roles in the SrtA activity. The residues I158,V168, L169, I199 and V201 form a hydrophobic pocket which interacts with Leu and Pro of the LPETG recognition site.[2] It has also been confirmed using mutagenesis studies that the residues H120 and R197 play an essential role during the reaction.[2] Using various studies, it has been established that the reaction of SrtA goes via reverse protonation mechanism.[7] Here, the imidazolium of the H120 residue acts as an acid whereas R197 helps in the stabilisation of the His-Cys dyad.

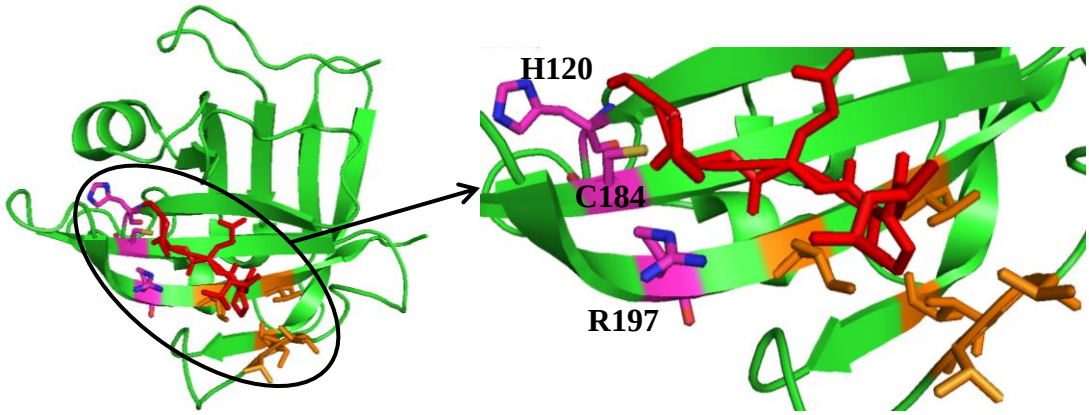

Figure 1.4: Crystal structure of $SrtA_{\Delta N59}$ (green) with LPETG (red), highlighting the active site of the enzyme. The C-terminus of the LPETG interacts with H120, C184 and R197 whereas the N-terminus interacts with the hydrophobic pocket (orange).

Kinetic studies of the SrtA enzymatic reactions have also been done. FRET assay to study the kinetics of SrtA has been carried out.[8] The substrate used is Abz-LPETG-Dnp and nucleophile used is triglycine. The data obtained from the experiment were fit to the rate equation:

$$v = \frac{k_{cat}\, ES}{S + K_m}$$

E= Enzyme concentration; S= Substrate concentration

The parameters obtained from the study are:

- $k_{cat} = 0.096 \pm 0.007\ min^{-1}$
- $K_m = 141 \pm 19\ \mu M$

In another study with a HPLC based assay of SrtA with Abz-LPETG-Dap(Dnp)-NH2 and Gly_5 as substrate resulted in the following kinetic parameters for the transpeptidation reaction:

- $k_{cat} = 0.27\ s^{-1}$
- $K_m = 5.5\ mM$

The discrepancy in the reported data has been explained by stating that SrtA is a more robust enzyme than previous data indicated.[9]

Trapping of the thioester-nucleophile intermediate can not only further validate the transpeptidation step but also illustrate the kinetics of the reaction.

Importance of studying reaction intermediates

Trapping of reaction intermediates is one of the challenging tasks, but an integral part to further our knowledge of chemistry. For studying reaction mechanism and kinetics, trapping of intermediates is a common method used. It is one of the most convincing evidence to prove that a particular reaction is in fact following a certain path.

Trapping of intermediates is also important for certain synthetic processes. There are cases where the synthesis of a desired product requires very harsh conditions, sometimes leading to poor/no yield of the products. For such reactions the approach of trapping

intermediates has been used,[10] as in the study mentioned, to carry out the reaction in milder conditions.

Another field where trapping intermediates has been found of utmost importance is in drug research. In several instances a marketed drug was withdrawn due to adverse drug reactions (ADRs) and idiosyncratic type adverse drug reactions (IADRs).[11] It has been hypothesized that most of the IADRs are related to reactive intermediates. Therefore, studying the formation of reactive intermediates forming in vivo that might be toxic in the earlier stages of any drug synthesis can help eliminate the chance of ADRs.

Conventionally, intermediates are trapped using methods like introduction of scavenger species into the reaction, or using time-resolved spectroscopic techniques like TCSPC (Time Correlated Single Photon Counting), etc. Other methods also include freeze quenching and X-ray crystallography, desorption electrospray ionisation mass spectrometry (DESI-MS).[12]

Using these methods apart from the confirmation of the formation of an intermediate, information like the average structural information,[13] rates of formation, electronic and vibrational energy transitions, [14] lifetime of transitions, can also be obtained.

In this thesis we report the use of Force Spectroscopy using Atomic Force Microscopy (AFM) to study a reaction taking place at a single molecule level. Here Force Spectroscopy is being used to trap an intermediate. The reaction taking place is being studied in real time. Using this experiment we can measure the exact off rates of the trapped intermediate. Also by using suitable nucleophile we can study how the nucleophilicity affects the lifetime of the intermediate.

The proposed method has a clear advantage over the other discussed methods because the reaction is being studied at a single molecule level rather than bulk. So, none of the measured parameters are being averaged over an ensemble, as what happens generally. This provides us with more accurate data.

Also, the lifetime measurement here starts at the exact moment the intermediate forms and lasts till just the decomposition. So, there is no loss of data. This method is, in some ways, analogous to watching the intermediate forming and decomposing in real time.

Chapter 2

Experiments, Results and Discussion

2.1 Introduction

To trap and study an intermediate in the enzymatic reaction of the SrtA enzyme, we attach the enzyme to the cantilever. Also, we attach one the reaction partner, the nucleophile to the coverslip, which serves as the surface for the experiment. Finally, we introduce the other reaction partner, the LPETGSSC peptide, in the reaction buffer. For the control experiment, the LPETGSSC peptide is not added to the buffer.

When the cantilever begins approaching the surface, it initially encounters the LPETGSSC peptide in the buffer, forming the thioester intermediate.[2] On further approach of the cantilever, the nucleophile comes in contact with the thioester intermediate. Our desired intermediate is formed. Then, the cantilever is retracted, pulling the trapped intermediate and we record the force measured using the AFM.

Using the experiment described above, we calculate the lifetime, off rates and the x_β values of the trapped intermediate. We can also study the effect of the change of the nucleophilicity if the attacking nucleophile on the trapped intermediate by changing the nucleophile attached to the surface.

2.2 Material and Methods

Functionalising of surface and cantilever

Glass coverslips and cantilevers were first plasma cleaned for 30 seconds in Plasma cleaner under a vacuum. Then they were cleaned using freshly prepared Piranha solution (3:1 volume ratio of concentrated H_2SO_4 and 30% H_2O_2) for 2 hours and 30 minutes respectively. The cantilevers were washed gently in milliQ water. The coverslips were first washed under flowing milliQ water and then sonicated in milliQ water at 53 kHz for 5 minutes. Following this, the coverslips were etched with KOH by sonicating the coverslips in 1M KOH for 15 minutes. Next, the coverslips were washed with milliQ water thoroughly followed by 3-4 cycles of sonication in milliQ water for five minutes each.

Silanization

For silanization of the coverslips and cantilever they were treated with 2% APTES (3-Aminopropyltriethoxysilane) in 95% Acetone solution for 30 minutes. Then they were

washed first with acetone, followed by milliQ water and again with acetone. Finally they were dried in vacuum oven at 110^{o}C for an hour.

PEGylation

Solutions of m-PEG-SVA and maleimide-PEG-SVA were prepared in PEGylation buffer (100mM $NaHCO_3$, 600mM H_2SO_4, pH 8.0) at a constant concentration of 0.1mg/μL. The coverslips were incubated with 10% of maleimide-PEG-SVA in mPEG-SVA for four hours.

For the cantilevers, PEGylation was done using 100% SVA-PEG-SVA solution in the PEGylation buffer at the same concentration of 0.1 mg/μL for four hours. Then the coverslips and the cantilevers were washed with water.

Introducing peptide to the surface

Following the PEGylation step, the coverslips were incubated in 100 μM of peptide (GGGGC/GRC/GDC) in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-7.5) for 7 hours at room temperature to immobilise the peptide onto the coverslip. After the incubation period, the coverslips were washed in water and stored in vacuum till use.

Attaching SrtA to the cantilever

The cantilever was incubated with 250nM SrtA in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, pH-8.0) for four hours. Next, it was washed by incubating in milliQ water. Then the cantilever was incubated in 2 μM glycine in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-8.0). Before starting the experiment, the cantilevers was incubated in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-7.5) for 3-5 minutes.

Single Molecule Force Spectroscopy of SrtA using Atomic Force Microscope (AFM)

SrtA was attached to the cantilever and polyglycine (GGGGC) was attached to the coverslip serving as the surface. The system was placed in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-7.5) containing 10μM LPETGSSC peptide. Different surfaces of 30μm * 30μm dimensions were chosen and raster scan of the selected area was performed. For each force scan the cantilever was

extended at a retracted at a constant velocity (1000nms^{-1}, 2000 nms^{-1}, 5000 nms^{-1}, 7500 nms^{-1}). As a control for the experiment buffer system containing no LPETGSSC peptide was used.

Cloning of SrtA-srttag

Polymerase Chain Reaction (PCR) amplification to introduce srttag

The plasmid with the srtA gene sequence was isolated from DH5α strain of *E. coli* bacteria using Qiagen DNA Mini Prep Kit. Primers required for the necessary mutation were designed. PCR was used to amplify the srttag incorporated insert. The amplified insert was then run on a 1% agarose gel and the expected DNA band was excised and purified using Qiagen DNA Gel Extraction Kit.

The **Table 2.1** lists the PCR mixture components and condition.

Initial denaturation	98°C	5mins	
Denaturation:	98°C	30secs	34 cycles
Annealing:	66°C	30secs	
Extension:	72°C	20secs	
	4°C	∞	
		Volume	
SrtA Template (97.26 ng/µL)		0.5 µL	
5X HF buffer		10 µL	
10 µM Forward Primer		2.5 µL	
10 µM Reverse Primer		2.5 µL	
dNTPs mix		0.4 µL	
Water		33.6 µL	
Phusion polymerase		0.5 µL	
Total		**50 µl**	

Table 2.1: PCR mixture components used for a single reaction. As a control for the reaction a mixture with no SrtA Template was placed under the same reaction condition.

Digestion of insert PCR product and vector

The amplified and purified PCR product and the vector (pET28a) were both double digested using the restriction digestion enzymes NdeI and BamHI at 37°C for 3hours following which the digested vector product was treated with CIP (Calf Intestinal Phosphatase) enzyme. Both the insert and the vector double digested products were then purified following agarose gel electrophoresis separation. The reaction mixture composition of the double digestion reaction is given in the **Table 2.2**.

Incubation time: 3 hrs

	Volume
Insert: srtA-srttag (30.023 ng/µL)	35 µL
10X 3.1 buffer	5 µL
Nde1	1 µL
BamH1	1 µL
Water	8 µL
Total	**50 µL**

Incubation time: 3 hrs (2hr before CIP treatment; 1hr after addition of CIP)

CIP added: 0.5 U

	Volume
Vector: pET28a (30.023 ng/µL)	10.5 µL
10X 3.1 buffer	5 µL
Nde1	1 µL
BamH1	1 µL
Water	32.5 µL
Total	**50 µL**

Table 2.2: Double digestion reaction mixture composition and conditions of a) insert: srtA-srttag b) vector: pET28a.

Ligation and transformation

The double digested vector and insert were ligated using T4 DNA Ligase enzyme at 16°C overnight. The ligation reaction mixture composition is given in the **Table 2.3**. The insert to vector molar ratio was kept at 3:1.

	Volume
Vector: pET28a (4.08 ng/µL)	11 µL
Insert: srtA-srttag (12.98 ng/ µL)	1 µL
10X T4 DNA ligase buffer	2 µL
T4 DNA Ligase	0.5 µL
Water	5.5 µL
Total	**20 µL**

Table 2.3: Ligation reaction mixture composition for srtA-srttag construct with insert: srtA-srttag and vector: pET28a. Here as a control experiment a self ligation reaction was carried out where no insert was added to the reaction mixture.

Following that, the ligation mixture was run on a 1% agarose gel to make sure that ligation had occurred. Next the ligation mixture was used to transform DH5α competent cells. To check positive transformation, colony PCR was carried out and the colony with the positive PCR result was sequenced for confirmation of positive mutation. Once confirmed, plasmids obtained from this colony were used for further transformed into both DH5α and BL21 (for protein expression) strains of *E. coli*.

Expression and purification of SrtA-srttag

The SrtA-srttag in pET28a plasmid was transformed into competent *E. coli* BL21 (RIPL) strain. The transformed cells were grown at 37°C in LB media till OD_{600} reached approximately 0.5 and then induced with Isopropyl- β-d-thiogalactopyranoside (IPTG) to a final concentration of 0.5mM. The cells were pelleted down after inducing the cultures at 25°C for 16hr. These pellets were processed for purification of SrtA-srttag. 5mL of resuspension buffer (50mM HEPES, 100mM NaCl, 50mM KCl, 2mM $CaCl_2$, pH 7.5) was added to re suspend the pellet after 3 freeze-thaw cycles. The bacterial cells were lysed under chilled conditions using probe sonication working at 20% amplitude for 5 minutes with 15sec on/off pulse. The lysed cells were centrifuged at 4°C for 20 minutes

and the supernatant was loaded onto a pre-equilibrated Ni-NTA resin for IMAC purification. Maximum amount of protein was eluted using 200mM Imidazole using step elution process. The eluted protein fractions were further purified using size-exclusion chromatography with Superdex 75 16/30 column. Purified protein was confirmed by analysing on SDS-PAGE.

Fluorescence imaging using TIRF microscope to confirm surface attachment of SrtA-srttag

In order to check the specific attachment of SrtA-srttag to a functionalised surface, the Cysteine-184 of SrtA-srttag is labelled with cy3-maleimide overnight. After removal of free dye by washing with buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-7.5) using a 3 kDa filter until the absorbance at 545nm (λ_{ex} of cy3) of the flow-through is negligible, the absorbance of the supernatant at 545 nm was measured and the concentration of the dye attached to proteins was calculated to ensure labelling ratio. A 1% PEGylated and polyG treated coverslip was first incubated with BSA for 30 minutes to prevent any non-specific attachment of protein. Then it was washed with buffer gently following which it was incubated in a mixture of SrtA-srttag and SrtA in the molar ratio of 4:5 for 1 hour. The coverslip was then gently washed with buffer before imaging using TIRF microscopy.

The obtained fluorescent spots were confirmed to be single molecules using single-step photo-bleaching.

Single Molecule Force Spectroscopy of SrtA-srttag using Atomic Force Microscope (AFM)

SrtA-srttag was attached to a 10% PEGylated and polyglycine treated cantilever using the SrtA reaction where SrtA-srttag to SrtA ratio was maintained at 4:5. Polyglycine (GGGGC), GRC or GDC peptides were attached to the coverslip serving as the surface. The system was placed in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, 10mM DTT, pH-7.5) containing 10μM LPETGSSC peptide. Different surfaces of 30μm X 30μm dimensions were chosen and force scan at a frequency of 1point/400 nm was performed. In the Force Ramp experiments, for each scan the cantilever was extended at a retracted at a constant velocity (1000nms^{-1}, 2000 nms^{-1}, 5000 nms^{-1}, 7500

nms^{-1}). As a control for the experiment buffer system containing no LPETGSSC peptide was used. The remaining experimental conditions were kept same.

In Force Clamp experiments, for each scan the molecules were pulled at constant force, keeping all the other experimental parameters constant.

2.3 Experiments and Results

We first attached the SrtA protein to the cantilever non-specifically. This cantilever was used for Force Ramp experiments. The schematic of the experiment is shown in the **Figure 2.1**.

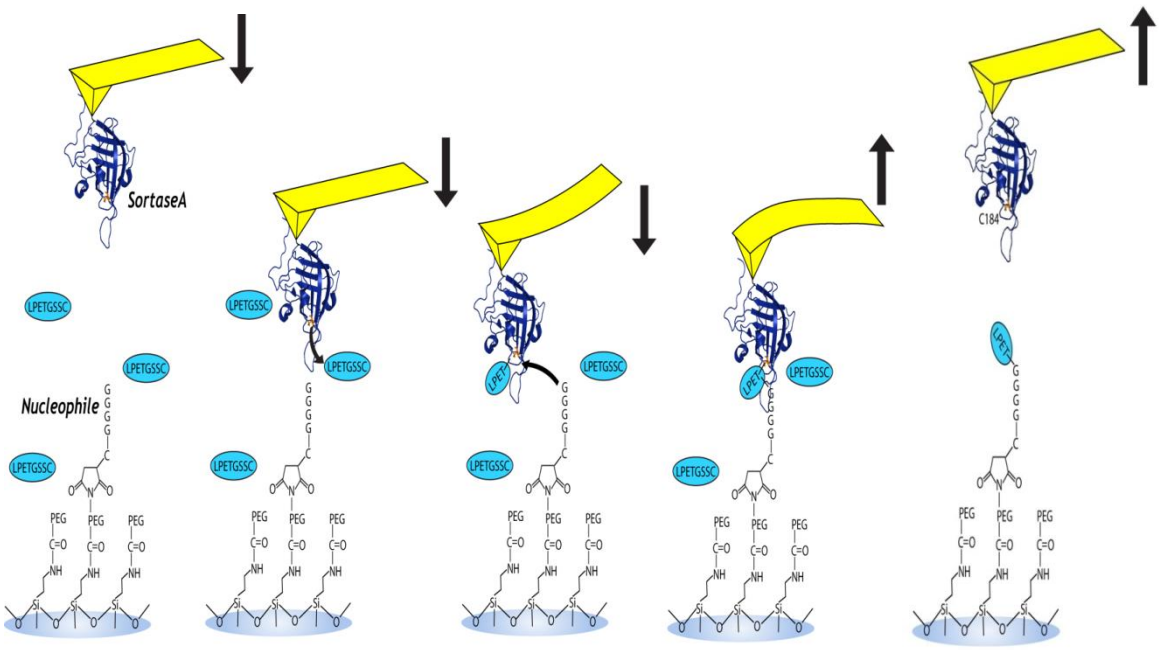

Figure 2.1: Schematic of the experiment of non-specifically attached SrtA using AFM. Here SrtA is attached non-specifically to the cantilever. When it reacts with the LPETGSSC present in the buffer, it forms the thioester intermediate. PolyG attached to the coverslip acts as the nucleophile to resolve this thioester intermediate.

The single stretching events obtained during Force Ramp experiment when SrtA was attached to the cantilever non-specifically confirmed the trapping of the desired intermediate. The data for different pulling velocities are plotted as histograms shown in **Figure 2.2**. The event percentage for the experiment is about (0.5-0.9%) which is low in comparison to the control experiment has ~0.3% events. So drawing any conclusion from these results could lead to artefacts. Thus the signal to noise ratio had to be optimised for any valid conclusions.

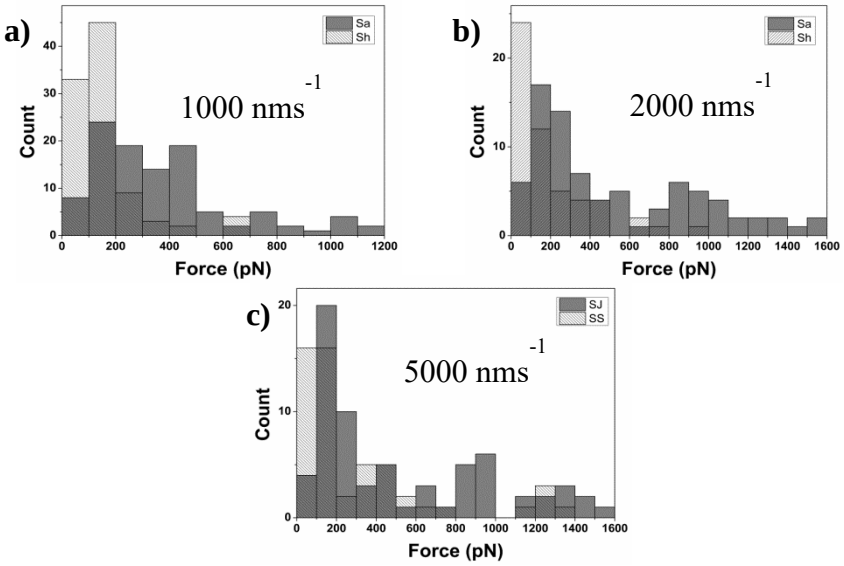

Figure 2.2: Histogram of unbinding force for different loading rates of the cantilever. a) 1000 nms^{-1} b) 2000 nms^{-1} c) 5000 nms^{-1}. The experiment repeats are labelled SS and SJ, respectively.

To increase the efficiency we have specifically attached the SrtA-srttag protein using sortagging on the cantilever, keeping the same experimental conditions. To clone SrtA-srttag we performed PCR to introduce the srttag as a mutation. Following the PCR the reaction mixtures were run on a 1% agarose gel (**Figure 2.3**).

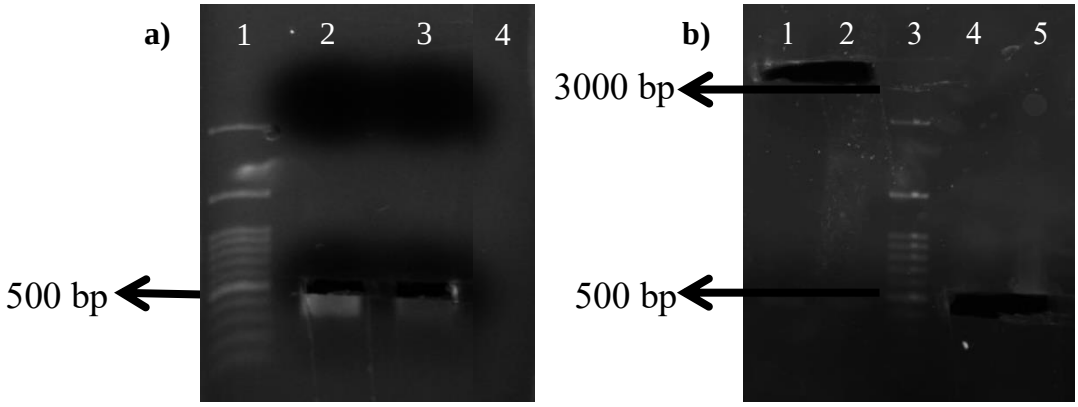

Figure 2.3: a) 1% agarose gel to confirm positive PCR product of SrtA-srttag. Lane 2 and 3 have the PCR product (expected size: 498 bp) whereas lane 4 has the negative control reaction mixture. The gel is run against a 100 bp DNA marker (Lane 1). b) 1% agarose to check double digestion using restriction enzymes Nde1 and BamH1 of pET28a vector (Lane 1 and Lane 2) and of SrtA-srttag insert (Lane 4 and Lane 5). The gel is run against 100 bp DNA marker (Lane 3).

After the gel extraction and purification of the PCR product double digestion of it was performed using the restriction enzymes Nde1 and BamH1 along with the double digestion of the vector, pET28a. The digested products were confirmed by running them on 1% agarose gel (**Figure 2.4**).

Next, we used the purified double digested insert (srtA-srttag) and vector (pET28a) to carry out the ligation reaction.

Post ligation, we did an agarose gel electrophoresis check to confirm ligation (**Figure 2.4 a**). Then this reaction mixture was used to transform DH5α competent cell of *E. coli*. After plating the cell on a LB agar plate (with 50μg/ml Kanamycin antibiotic) and incubating the plates at 37°C for 14-16 hours 5 colonies grew on the plate with cells transformed by the ligation mixture opposed to the plate with the cells transformed with the self-ligation mixture, in which no colonies were observed.

For the positive confirmation of the insertion of the insert into the vector, colony PCR of the 5 obtained colonies was done. The PCR reaction conditions were kept the same as the table. The agarose gel electrophoresis using 1% agarose gel (**Figure 2.4 b**) confirmed that all the 5 colonies had the vector with the insert. Next, from 1 of these colonies plasmids were isolated and used to carry out transformation of both DH5α and BL21-RIPL strains of *E. coli*. These plasmids were also sequenced to confirm the desired mutation.

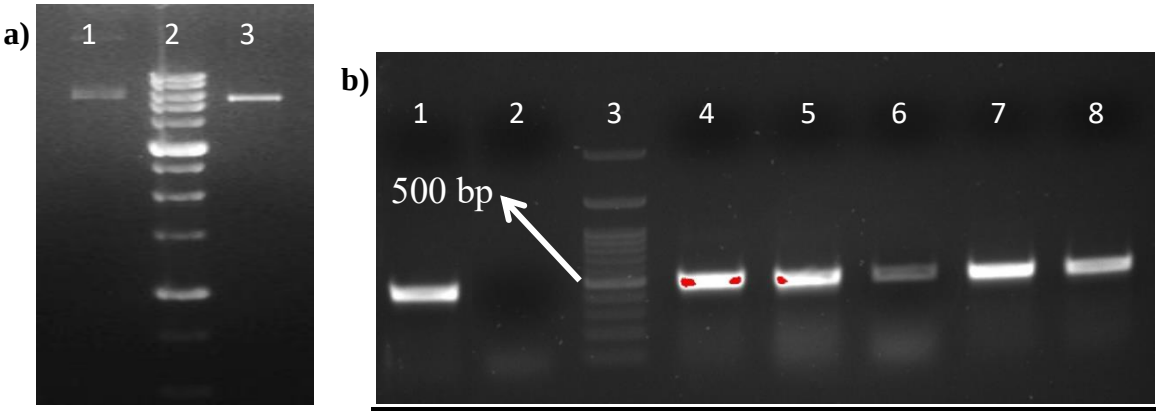

Figure 2.4: a) 1% agarose gel to check ligation of insert (srtA-srttag) and vector (pET28a). Lane 1 (ligation mixture) shows a smear pattern confirming ligation whereas lane 3 (digested product) shows no such smear. Lane 2 contains 1kb DNA marker. b) 1% agarose gel after colony PCR. Here lane 1 is normal PCR product of srtA-srttag, lane 2 is the negative control product for the PCR whereas lanes 4 to 8 are the colony PCR product of the 5 colonies obtained. The expected product in all 5 colony PCR is of 498bp.

Next, we expressed and purified the protein SrtA-srttag using the transformed BL21-CodonPlus (DE3)-RIPL cells. After purification via IMAC and SEC, we observed a protein band at ~50kDa (possible dimer) when the fractions were run on a SDS PAGE (**Figure 2.5 a**) which could not be resolved using SEC. To remove this band we incubated the purified protein in buffer (50mM HEPES, 150mM NaCl, 50mM KCl, 2mM $CaCl_2$, pH-7.5) with 1mM DTT and 10mM Cysteine for 1 hour at 37°C. Following this, we dialysed out the DTT from the buffer and purified the protein sample using SEC. SDS PAGE (**Figure 2.5 b**) of the pure fractions thus obtained confirmed the removal of the dimer from the sample.

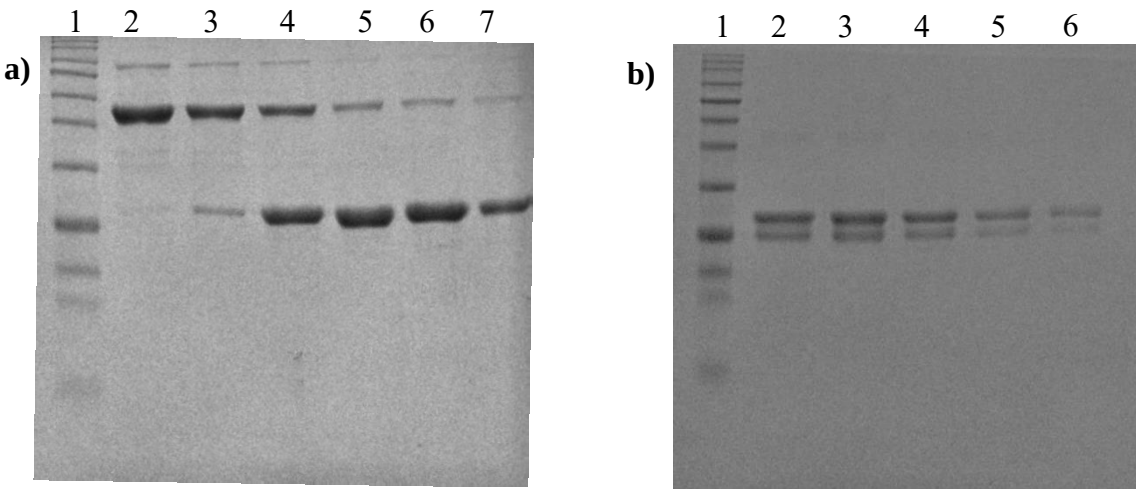

Figure 2.5: a) 12% SDS gel for SrtA-srttag protein after IMAC and SEC purification. Lane 2-7 contain different fraction collected after SEC. b) 12% SDS gel after DTT/ Cys treatment and SEC. Lane 2-6 are different SEC fractions collected. Lane 1 in both a) and b) shows the pre-stained protein marker used.

For confirming the correct orientation of the protein attaching on the surface at single molecule level, we performed experiments using TIRF microscope. The protein (labelled using Cy3-maleimide) was attached to the surface and imaged by exciting the molecules using a 480nm laser source (**Figure 2.6 a**). Then, we did photo-bleaching experiments to confirm the single molecule level attachment (**Figure 2.6 b**).

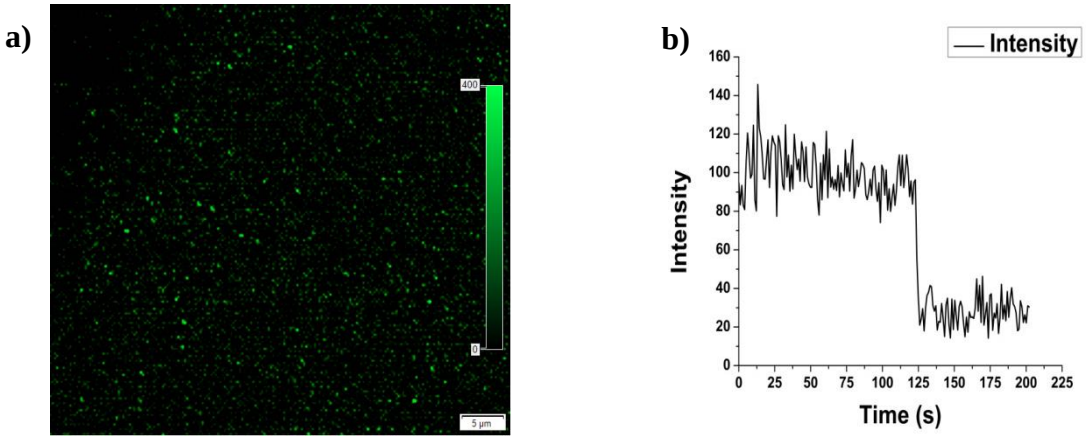

Figure 2.6: a) TIRF image of SrtA-srttag protein attached to a 1% PEGylated surface. b) The intensity profile showing single step photo-bleaching event.

Next, we performed single molecule force spectroscopic experiments for understanding the kinetics of the trapped intermediate. A single force curve event was observed when the SrtA-srttag on the cantilever (**Figure 2.7**) first formed a thioester intermediate with the LPETGSSC peptides present in the buffer. Then, on bringing it further down the terminal amine group of the polyglycine attacked the thioester bond and our desired intermediate formed.

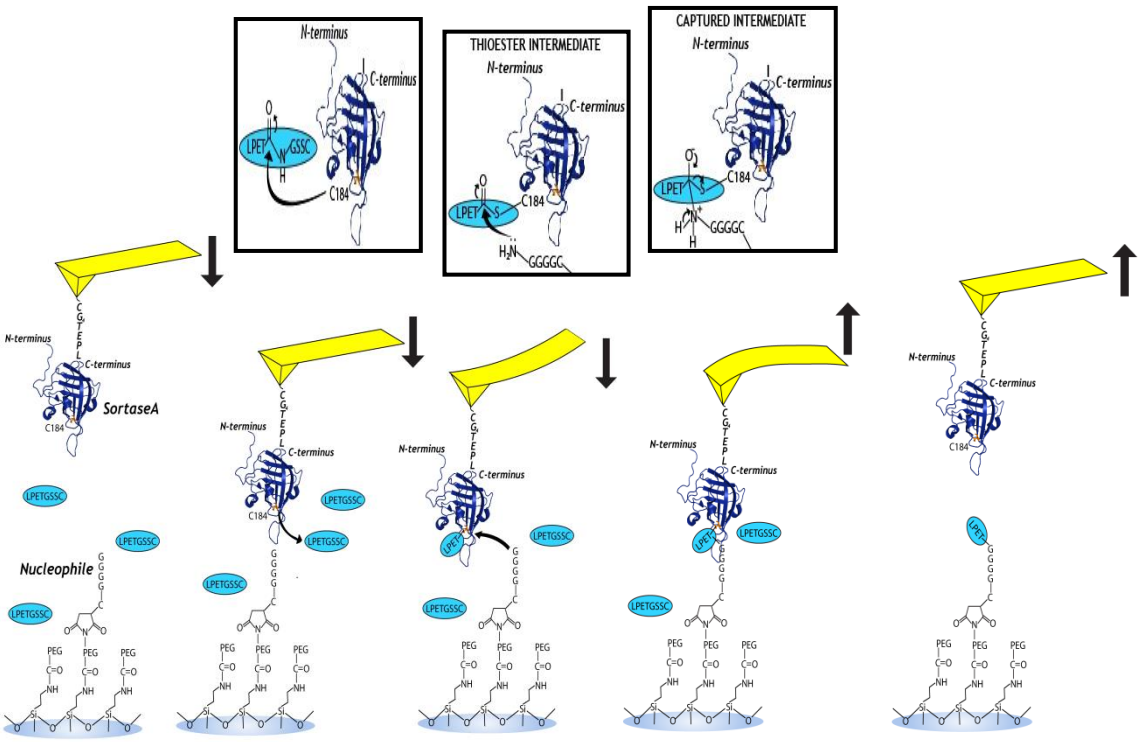

Figure 2.7: Schematic of the AFM force spectroscopy experiment performed. The inset images further illustrate the specific reaction taking place at the particular step.

We did a force ramp experiment using AFM with different pulling velocities. Histograms of the force distribution for the different pulling velocities were plotted and fitted to a normal distribution (**Figure 2.8**). Also, the event ratio of the experiment was between 1.7-2.0% events, in contrast to the control experiment (without LPETGSSC peptide in the buffer) which yielded an event ratio of 0.42%.

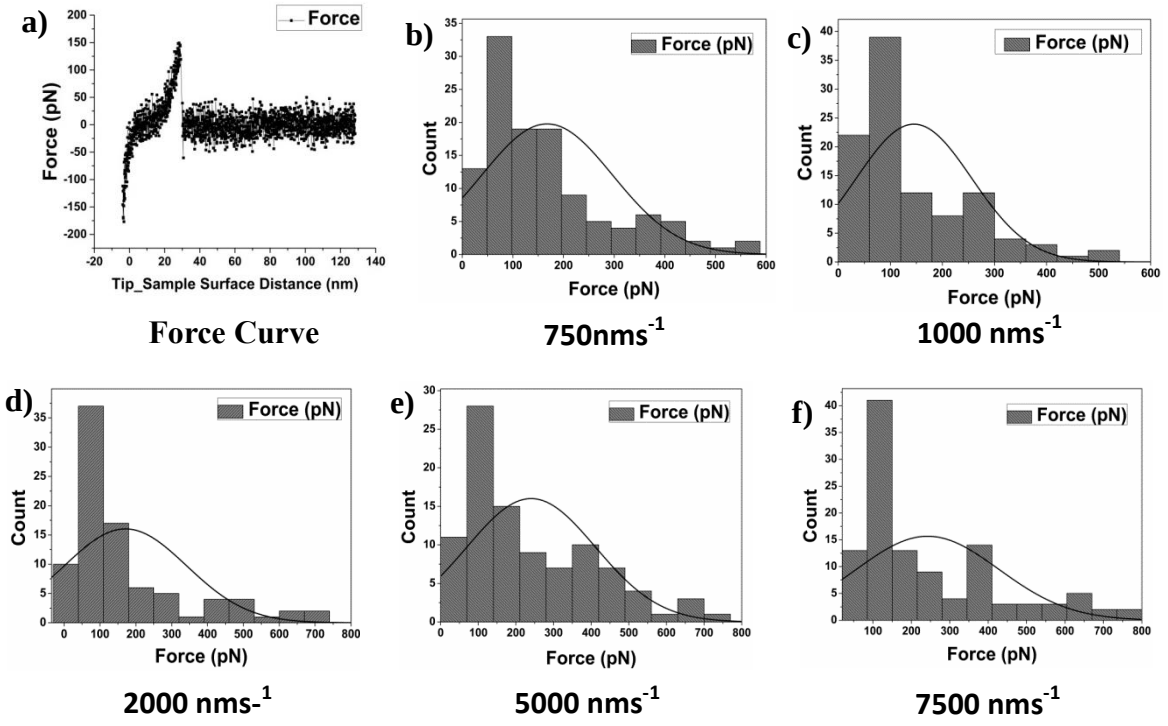

Figure 2.8: a) Typical force curve for a single molecule event when an intermediate is potentially captured. Histograms of force for pulling velocities: b) 750nms^{-1} c) 1000nms^{-1} d) 2000nms^{-1} e) 5000nms^{-1} f) 7500nms^{-1}. Each histogram is fitted to a normal distribution curve.

Using the most probable unbinding forces obtained from the force distribution of each pulling velocity, we plotted a force versus ln (loading rate) graph. Using the Bell-Evans equation to fit this graph (**Figure 2.9**), we calculated the lifetime of the intermediate to be 6.0 ± 0.4ms. We also calculated the k_{off} of the reaction to be 166.67 ± 9.8s^{-1}, and x_β (the distance between the reactant and the transition state in the potential energy diagram of the reaction) for the captured intermediate came out to be 0.0751 ± 0.0007nm. The following is the Bell-Evans equation used:

$$F = \frac{k_B T}{x_\beta} \ln\left(\frac{\tau_o x_\beta (LR)}{k_B T}\right)$$

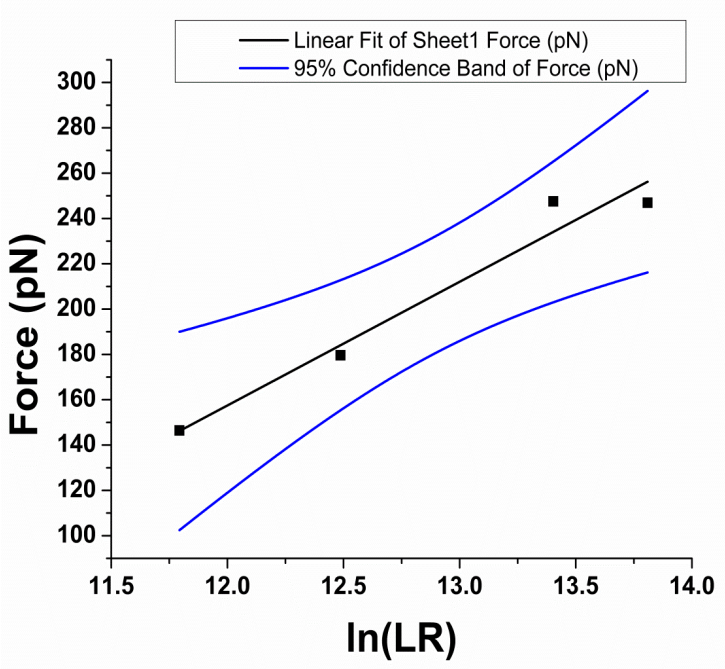

Figure 2.9: Graph of Force versus natural log of Loading Rate. Here forces for different loading rates (force/time) are the most probable force obtained from the force distribution. The points are fitted linearly and the Bell-Evans model is used to calculate the lifetime, off rates and x_β of the intermediate.

2.4 Conclusion

From the presented results, it can be concluded that we have been successful in capturing the intended intermediate. The initial experiment scheme used yielded very low event percentages and low signal to noise ratio. It was hypothesized that this was due to the attachment of the SrtA enzyme non-specifically to the cantilever, which could have led to an orientation of the enzyme unfavourable to the occurrence of the reaction. This could have, in turn led to a decreased efficiency of the overall experiment.

To overcome this limitation, it was decided to attach the SrtA enzyme specifically to the cantilever, making sure the optimum orientation for the reaction to take place. A srttag at the C-terminus of the enzyme was incorporated. This led to an increase in the overall event percentage as well as the signal to noise ratio of the experiment over the control experiment. Using the data obtained from the Force Ramp experiments performed, we calculated the lifetime, off rates and the x_β of the captured intermediate.

Next, we want to perform experiments using higher percentage of PEGylated surface to increase the percentage of events further, so as to get a unbinding force distribution which fits better to a normal distribution. We also want to perform Force Clamp experiments for the captured intermediate to get further lifetime information. Finally, we wish to study the effect of nucleophilicity on the captured intermediate. To do that we want to use different nucleophiles instead of polyglycine. For some of these experiments, the initial data collection process is done.

Bibliography

(1) Fey, P. D.; Rupp, M. E.; Hinrichs, S. H.; Davis, C. C.; Kreiswirth, B. N.; Schlievert, P. M. *Antimicrob. Agents Chemother.* **2003**, *47* (1), 196–203.

(2) Clancy, K. W.; Melvin, J. A.; McCafferty, D. G. *Biopolymers* **2010**, *94* (4), 385–396.

(3) Maresso, A. W.; Schneewind, O. *Pharmacol. Rev.* **2008**, *60* (1), 128–141.

(4) Ritzefeld, M. **2014**, 8516–8529.

(5) Ritzefeld, M.; Sewald, N. *J. Amino Acids* **2012**, *2012*, 816032.

(6) Ton-that, A. H.; Liu, G.; Mazmanian, S. K.; Faull, K. F.; Ton-thatt, H.; Liut, G.; Mazmaniant, S. K.; Faull, K. F.; Schneewindt, O. **2016**.

(7) Frankel, B. A.; Kruger, R. G.; Robinson, D. E.; Kelleher, N. L.; McCafferty, D. G. *Biochemistry* **2005**, *44* (33), 11188–11200.

(8) Huang, X.; Aulabaugh, A.; Ding, W.; Kapoor, B.; Alksne, L.; Tabei, K.; Ellestad, G. *Biochemistry* **2003**, *42* (38), 11307–11315.

(9) Kruger, R. G.; Dostal, P.; McCafferty, D. G. *Anal. Biochem.* **2004**, *326* (1), 42–48.

(10) Kristensen, J.; Lysén, M.; Vedsø, P.; Begtrup, M. *Org. Lett.* **2001**, *3* (10), 1435–1437.

(11) Orhan, H.; Vermeulen, N. P. *Curr. Drug Metab.* **2011**, *12* (4), 383–394.

(12) Perry, R. H.; Splendore, M.; Chien, A.; Davis, N. K.; Zare, R. N. *Angew. Chemie - Int. Ed.* **2011**, *50* (1), 250–254.

(13) Schlichting, I.; Chu, K. *Curr. Opin. Struct. Biol.* **2000**, *10* (6), 744–752.

(14) Jacox, M. E. *Int. J. Mass Spectrom.* **2007**, *267* (1–3 SPEC. ISS.), 268–276.