

Examining the Behavior of Surface Tethered Enzymes

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Chemical Biology)
in the University of Michigan
2015

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Chapter 1

Introduction

1.1 Introduction

1.1.1 Applications for Immobilized Enzymes

Enzymes catalyze stereospecific chemical reactions under mild conditions. Immobilized enzymes have been utilized and proposed for a variety of applications, from medicine to environmental remediation, to environmentally benign catalysts for the production of industrial chemicals. One of the most common applications for immobilized enzymes is in the development of biosensors¹. One example is the immobilization of glucose oxidase for the detection of blood glucose. In this case, glucose oxidase converts glucose to gluconolactone and hydrogen peroxide, a reaction that can be followed using a platinum probe. In addition, detection assays have been developed using cholesterol oxidase and uricase for the measurement of cholesterol² and uric acid concentrations³, respectively. Antimicrobial peptides such as MSI-78 and esterases such as subtilisin have been proposed to be attached to surfaces to prevent the formation of biofilms⁴, particularly for implantable materials.

Environmental treatment and remediation is another application. Because of its ability to oxidize a broad variety of phenols, laccases are commonly used to treat wastewater effluent⁵. More recently, the ability of carbonic anhydrase to hydrolyze CO₂ has been explored as a method for carbon capture⁶.

More recently, researchers have been exploring the use of immobilized enzymes for the development of biofuels. For example, lipases are commonly used to generate biodiesel through the hydrolysis of lipids into fatty acid alkyl esters of various chain lengths^{7,8}. This process

allows for the production of fuel from a variety of plant oils. Alternatively, cellulases have been used in the hydrolysis of cellulose and other polysaccharides into monosaccharides that can be fermented to produce bioethanol.

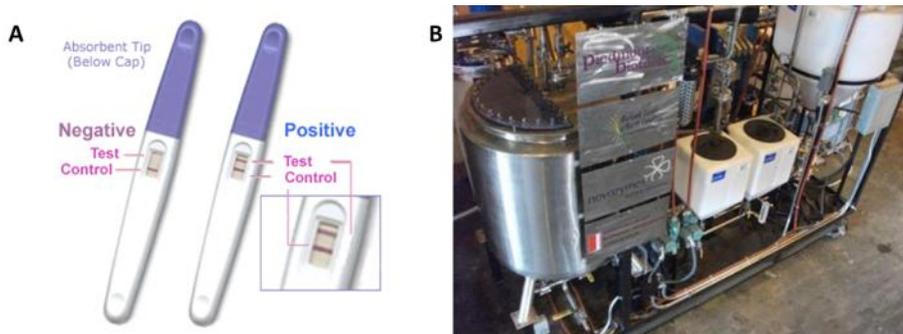


Figure 1.1: Applications of immobilized proteins include A) Biosensors and B) Industrial catalysts

Immobilized enzymes have been proposed for use in other industrial processes. Penicillin Acylase, for example, is a hydrolase capable of producing the chemical intermediate involved in the synthesis of side-chain modified penicillins (e.g. ampicillin, amoxicillin, cloxacillin, salbactam) and cephalosporins (cephadroxil, cefalexins)⁹. Phenylalanine ammonia lyase has been proposed for the deamination of phenylalanine to cinnamic acid, a precursor in the formation of styrene¹⁰.

1.1.2 Advantages and Disadvantages of Enzyme Immobilization

Most commonly, enzymes are immobilized to facilitate recapture and recycling¹¹. Improving the recapture of the enzyme will lead to improved purity of the product, minimizing protein contamination and decreasing manufacturing costs. Recycling the enzyme can help increase the total product yield of the process, further decreasing costs.

In addition to the economic benefits, it is widely claimed that immobilization can increase stabilization¹², usually through a variety of poorly understood and largely unsubstantiated mechanisms. It is hypothesized that immobilized enzymes have an increased resistance to thermal unfolding because of the restricted range of motion. Resistance to proteases and organic solvents have been speculated to be caused by the restricted access of the bulk solution to the surface tethered enzymes. Others, however, have found that immobilization can lead to destabilization¹³. The molecular mechanisms governing these behaviors are still not well understood.

It has often been observed that the specific activity of enzyme decreases when the enzyme is tethered to a surface¹⁰⁻¹². This has been explained using a variety of hypotheses, including unfavorable electrostatic interactions and the rigidity of the protein tertiary structure. The amount of activity loss is also often dependent on how the protein is tethered to the surface – randomly adsorbed enzymes have been shown to have the largest drop in specific activity, followed by random covalent cross-linking, while enzymes tethered via a single, directed covalent attachment generally tend to retain the higher specific activity¹². A detailed molecular level understanding of activity loss, however, is still lacking.

1.2 Protein Surface Interactions

1.2.1 Introduction

There are a variety of methods to immobilize an enzyme on a surface, including adsorption, covalent attachment, and encapsulation. Because of the added complexity of diffusion of substrate through the material, encapsulation will not be considered here.

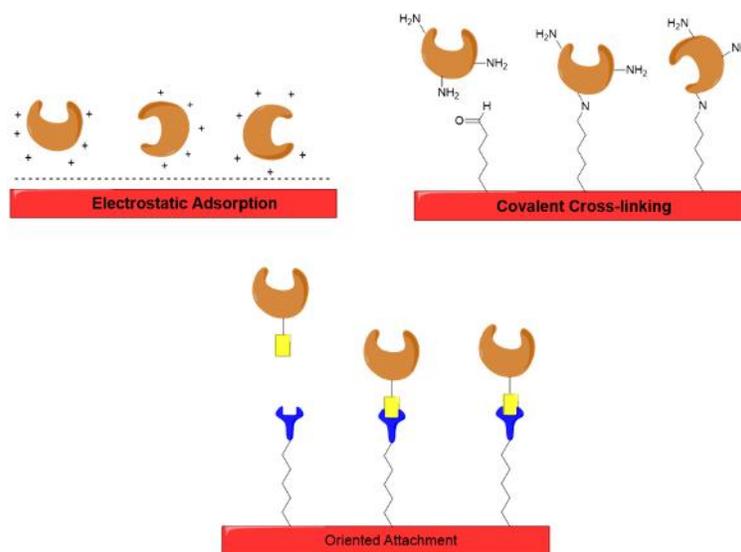


Figure 1.2: Various methods to immobilize enzymes on surfaces

Adsorption tethers the protein to the surface through non-covalent interactions, utilizing electrostatic and van der Waals forces. Adsorption has the benefit of requiring no modification to the enzyme, and can be undertaken in aqueous conditions. The weak protein-surface interactions, however, means that protein can diffuse from the surface over time. In addition, the non-specific nature of the attachment allows for multiple attachment points that can frequently lead to partial or complete unfolding, resulting in a loss of activity.

Covalent tethering prevents the protein from diffusing from the surface. The simplest way to accomplish this is to use cross-coupling reactions that exploit readily available chemical groups on the enzyme surface, such as amines and carboxylic acids. Similarly to non-covalent protein adsorption, however, the non-specific nature of this method can lead to unfolding by covalently tethering the immobilized protein in non-native conformations.

Another approach is to functionalize the protein with a bioorthogonal functional group that will react specifically with a complementary functional group present on the surface of interest¹⁴. Such site-specific immobilization provides an ordered orientation of the enzyme and prevents the unfolding that can result from non-specific immobilization. For example, Kalia *et al.* tethered an azido group to the C-terminus of bovine pancreatic ribonuclease. This modified protein was tethered to a phosphinothioester-modified monolayer. Because such protein modifications rely on N- or C- terminal modifications of the enzyme, directed orientation is limited.

1.2.2 Adsorption

Proteins adsorb to surfaces either because of electrostatic interactions or because of favorable hydrophobic interactions. Although some have looked to adsorption as a possible mechanism for derivatizing a surface with an enzyme, the weak surface interaction and non-specific nature of attachment has led most researchers to look to covalently attach a protein to a surface. Instead, the vast majority of research regarding protein adsorption is in the field of implantable biomaterials, where the goal is to prevent nonspecific interactions between the implanted device and surrounding biochemical molecules. Still, the research provides interesting insights that can lead towards the development of surfaces that minimize potentially destabilizing interactions with the tethered enzyme.

Although the direct interaction between the protein and the surface is an important factor in adsorption, it is the interaction between the solvent (typically buffered water) and the material interface that will drive protein adsorption¹⁶, caused both by the energy necessary to displace water molecules from the surface and the entropic gain from releasing the water into the bulk solution. Xu and coworkers¹⁷ exposed polyethylene to glow discharge plasma for different periods of time to expose differing amounts of hydroxyl groups on the polymer surface, proportional to the exposure time. The resulting hydrophilicity was quantified by measuring water contact angle, a measure of the angle between a droplet of water and the contact surface. The more hydrophilic the surface, the more likely the droplet of water is to spread out, and thus the smaller the contact angle as seen in figure 3. A positive correlation was observed with surface wettability – the more hydrophobic the surface, the stronger the adhesion forces of the adsorbed proteins, as measured using AFM.

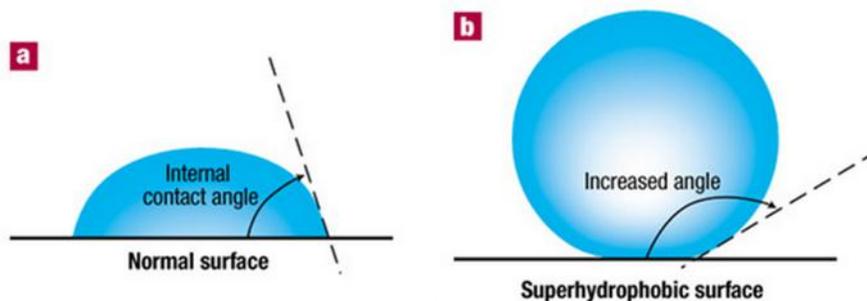


Figure 1.3: Measuring hydrophobicity using water contact angle (as adapted from Nature Materials 1, 14 – 15 (2002))

For hydrophobic materials, the water molecules remain ordered near the surface¹⁸. The increase in entropy from the release of these ordered water molecules to the bulk is considered to be the driving force for strong adsorption of proteins to hydrophobic surfaces¹⁸. Because of

favorable interactions with the hydrophobic residues in the protein core, adsorption onto hydrophobic surfaces often leads to a significant loss of activity.

One notable exception, however, is lipase which has demonstrated an increase in specific activity upon adsorption to hydrophobic nanoparticles¹⁹. This increase has been hypothesized to be caused by an active site flap on the protein being stabilized in the open conformation, thus allowing substrate to more easily diffuse into and out of the active site.

For hydrophilic surfaces, surface waters can be repulsive. It is hypothesized that disrupting the water-surface interaction can be too energetically costly, thus preventing nonspecific adsorption^{18,20}. Using atomic force microscopy, Valle-Delgado and coworkers measured an additional repulsive force, one that was proportional to the ionic strength of the solution. This force was attributed to hydrated ions that form a double layer at the material interface.

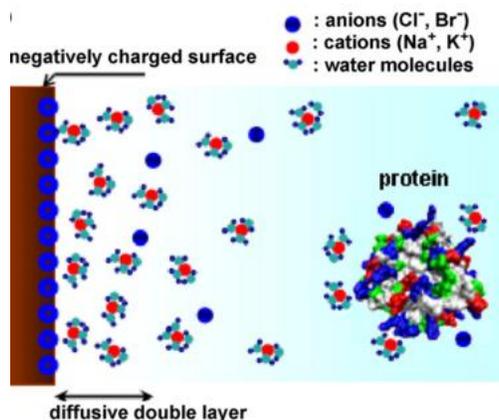


Figure 1.4: A depiction of the ionic double layer that forms at the material interface. Taken from *Materials Science and Engineering B 152* (2008) 2–7

In order to establish a set of rules from which to design surfaces that are inert to protein adsorption, Chapman et. al²¹ derivatized gold surfaces with long chain carbon monolayers

terminated with chemical moieties that varied in hydrophilicity. Surface plasmon resonance was used to rapidly examine the degree of protein adsorption. As expected, hydrophilic surfaces were found to resist protein adsorption while hydrophobic surfaces displayed a propensity towards protein aggregation. Interestingly, however, it was found that surfaces containing hydrogen bond donors could occasionally promote a small degree of adsorption. It was hypothesized that the surface was competing with water and structural hydrogen bonds on and within the protein.

Herrwerth et al²² extended this research to look not only at the hydrophilicity of the terminal groups, but also to the hydrophilicity of the monolayer chains themselves. They derivatized surfaces with polypropylene (more hydrophobic) and polyethylene glycol (more hydrophilic), terminated with either a methyl or hydroxyl group, and studied the amount of fibrinogen that adsorbed. They found that to minimize protein adsorption, both the terminal chemical moiety and the linker chain needed to be hydrophilic. If either of the two were hydrophobic, the propensity for protein adsorption increased.

An intriguing follow-up study was published by the Santore group²³. This group coimmobilized PEG with polylysine to create a surface with positively charged patches intermingled with PEG, a polymer known for resisting non-specific protein adsorption. By varying the ratio of polylysine to PEG used for immobilization, these researchers controlled the mean size of the charged patches. Using AFM, they were able to demonstrate the footprint of the adsorbing fibrinogen positively correlated with the expected size of the polylysine patches. In addition, they observed that if the polylysine patch was too small, no adsorption occurred at all.

In addition to hydrophilicity, researchers have also examined the role of charge on protein surface interactions. For example, Pasche and coworkers examined the interaction between protein charge, surface charge, and the ionic strength of the bulk solution²⁴. As expected, it was found that the positively charged lysozyme was attracted to the negatively charged surface while the negatively charged α -lactalbumin adsorbed onto the positively charged surface. Interestingly, they found that though the solution ionic strength played little role in regulating adsorption onto charged surfaces, increased ionic strength seemed to decrease adsorption onto the non-polar hydrophilic PEG surface. This was attributed to the formation of an ionic double layer at the material-bulk solution interface. Similarly, Feller et al²⁵ found that increased ionic strength can lead to decreased adsorption onto charged surfaces.

To explore the role of surface charge on adsorption further, researchers began examining what effect a zwitterionic monolayer would have on protein-surface interactions. It was hypothesized that zwitterionic surfaces would have a higher resistance to adsorption than non-polar hydrophilic surfaces²⁶, since electrostatic interactions would bind water molecules more tightly to the surface than by hydrogen bonding. While this hasn't always proven to be the case experimentally, zwitterionic surfaces have been shown to be at least as resistant to non-specific adsorption as their non-polar hydrophilic counterparts. Tegoulia *et al.* demonstrated that the zwitterionic phosphatidylcholine monolayer was as resistant to fibrinogen adsorption as hydroxyl terminated monolayers²⁷.

1.2.3 Covalent tethering

There are a number of techniques that can be used for covalently tethering proteins to surfaces. The most basic technique involves the use of a surface-bound chemical moiety that is

capable of crosslinking to a functional group on the surface of the protein. Examples include amine-reactive groups such as N-hydroxy-succinimide¹⁹, carboxylic acid reactive groups such as carbodiimides¹⁹, and hydroxyl-reactive groups such as epoxides. Although these reactive groups are readily available on the surface of the protein, the non-specific nature of these reactions have a high probability of resulting in unfavorable orientations and conformations for the tethered protein.

More recently, researchers began studying attachment techniques that result in a single unique attachment. These approaches often require the modification of the protein to accommodate the necessary reactive group. In one case, a conjugated diene is covalently attached to the enzyme – usually at one of the termini – and undergoes a Diels-Alder reaction with a surface bound dienophile such as benzoquinone²⁸. A similar approach can be taken with azido- and alkynyl groups undergoing “click chemistry”. Native chemical ligation uses the reaction between an N-terminal unprotected cysteine and a surface bound thioester to form a peptide bond with the surface linker.

While these approaches will effectively introduce a single attachment point between the enzyme and the surface, they limit the attachment chemistry to one of the two termini, thus limiting the available orientations, or require the introduction of non-native amino acids, which can suffer from low incorporation rates and the potential perturbation of the native structure. To overcome these issues, researchers have started examining thiol maleimide chemistry as a means for attaching biomolecules to surfaces²⁹. Maleimide chemistry has long been used for site specific bioconjugation of small molecules to proteins³⁰. The reaction is specific under physiological conditions, allowing the reaction to occur under conditions that will not denature the protein of interest.

Regardless of the attachment chemistry, very little experimental work has been done to elucidate molecular level detail protein-surface interactions under these conditions. Some molecular dynamic simulations, however, have been done to start addressing these questions. For example, Wei and coworkers began developing a coarse grain model for exploring the effect of a single attachment site on the activity and stability of immobilized enzymes³¹. The surface was modeled to be inert, acting as mildly repulsive to the enzyme. The tethering site was modeled to be a single spring attached at various surface-exposed residues on the enzyme. The enzyme was modeled at different temperatures, using the number of native structural contacts (hydrogen bonds, salt bridges) relative to the crystal structure as a measure of structural activity. They found that the structural integrity of an enzyme was more likely to be retained if the enzyme maintained a higher degree of freedom of motion. Attachment sites that seemed to restrict this freedom of motion resulted in a decrease in the thermostability of the surface tethered enzyme. They hypothesized that this decrease in stability resulted from the energy that would otherwise have gone into the freedom of motion of the enzyme being instead consumed by the breaking of structurally essential interactions within the protein.

1.3 Techniques for Studying Surface Tethered Enzymes

1.3.1 Introduction

Because of the low concentrations of protein available on surfaces, examining the structural integrity of surface tethered enzymes is quite difficult. Traditional methods for studying secondary and tertiary structure, such as crystallography and solution NMR, cannot be used to assess proteins attached to solid interfaces. Although enzyme activity is one possible indirect method for assessing the structural integrity of surface tethered enzymes, other

methodologies have been developed³², including surface plasmon resonance, ellipsometry, atomic force microscopy, solid state NMR, and Sum Frequency Generation. Each of them is briefly described.

1.3.2 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) has long been used to assess the activity of enzymes tethered to gold surfaces. SPR uses a light source to initiate oscillations of electrons on the gold surface. The frequency of the light necessary to initiate the oscillation is dependent on the restoring force of the nuclei, and can also vary as the surface is chemically modified. This last feature makes SPR a sensitive technique for studying the adsorption of enzymes. One of the benefits of using this technique is that the enzyme does not need to be modified in any way. In addition, the resonance frequency has been reported to be sensitive to conformational changes of receptor during ligand binding, making this a common tool to assess the kinetics of receptor-ligand binding. A limitation, however, is that SPR provides no information about secondary structure.

1.3.3 Ellipsometry

Ellipsometry is a common technique used to study thin films. The change in polarity of light reflected from the surface changes with the formation and growth of thin films. This change is proportional both to the thickness as well as the refractive index of the film, making this technique useful for measuring the rate of film formation. Like SPR, no labeling or modification of the protein is required. However, also like SPR, little information about secondary structure can be extracted.

1.3.4 Atomic Force Microscopy

Another common technique for assessing surfaces is Atomic Force Microscopy, or AFM. AFM uses a cantilever with a micrometer sized tip to assess the topology of surfaces. As the tip moves across the surface, changes in the height of the surface leads to a displacement of the cantilever. This displacement is measured using a laser reflected from the cantilever. By modifying both the flat surface and the tip, either through attaching polymeric monolayers or through the adsorption of enzymes, AFM can use changes in cantilever displacement to measure relevant attractive and repulsive forces. Because of this, AFM is often used to assess the relevant forces present at material interfaces as well as topology.

1.3.5 Solid State NMR

Unlike the previously mentioned techniques, solid state NMR can provide structural information concerning surface-tethered biomolecules. Like solution NMR, solid state NMR looks at nuclear spin in the presence of a magnetic field. However, the measured chemical shifts, influenced by structural neighbors within the biomolecule, are also influenced by the surface itself, both by the chemical identity of the surface as well as the orientation of the molecule relative to the interface. While successfully applied to amyloid formation and membrane proteins³³, one problem that solid state NMR faces is low sensitivity, making a detailed study of surface-tethered enzymes challenging.

1.3.6 Sum Frequency Generation

Sum Frequency Generation is an IR-based spectroscopic technique that is sensitive and specific to the surface. The principle is based on the idea that at interfaces where there is no inversion symmetry, the frequencies of two photons fired at the surface will add together, producing a characteristic third photon. Typically, one photon is in the IR region and acts as a probe of chemical structure, while the other is in the visible spectrum.

It is usually the vibrations of the amide proton and the peptide carbonyl that is measured with SFG. The vibrational spectra of these constituent groups are sensitive to secondary structure. Because of the net dipole of the α -helix generated by these bonds, the orientation of a helix can be determined relative to the surface through the use of polarized light. For enzymes where the tertiary structure aligns to generate a net dipole, the orientation of the enzyme can also be determined relative to the surface, assuming that the crystal structure of the protein is available. For these reasons, SFG is a powerful technique for assessing the orientation and distribution of surface tethered enzymes.

1.4 Goals of the Project

The goal of my research was to explore in more detail the mechanisms that underlie the changes in activity and stability observed upon enzyme immobilization. I have engineered a number of *L. lactis* derived β -galactosidases, each with a unique surface exposed cysteine. Taking advantage of the specificity of thiol-maleimide chemistry to immobilize the enzymes to surfaces with defined orientations, allowed the role of orientation and location of tethering site to be examined. I used a PEG-derived monolayer functionalized with a variety of terminal chemical moieties to explore the role of electrostatics and hydrodynamics on the specific activity and thermal stability. In collaboration with the Chen lab, surface specific IR technique Sum

Frequency Generation was to determine the orientation of the surface bound enzyme, and compared this with the predicted orientation. In collaboration with the Brooks lab, coarse grain simulations were performed in an effort to elucidate a more detailed chemical mechanism.

In chapter 2, we established the model system. Four constructs were generated, each with a unique, solvent-exposed cysteine. These cysteine residues allowed us to tether α -gal to maleimide-derivatized surfaces via a unique chemical linkage. In solution the four constructs have k_{cat} and K_m values that are within error of one another and within error of the published value for wild type α -gal. In collaboration with the Chen lab, we successfully used Sum Frequency Generation (SFG) and ATR-FTIR to experimentally determine the orientation of immobilized V152C.

In chapter 3, we examined the effect that the attachment site has on the thermal stability of immobilized α -gal. Two constructs of α -gal were tested, each containing a unique, solvent-exposed cysteine distal to the active site: V152C and E147C. The V152C construct introduces a cysteine residue into a loop distal to the active site. The E147C construct introduces a cysteine residue into a helix distal to the active site. The cysteine residues of these two constructs were covalently reacted with maleimide-derivatized glass beads, immobilizing the proteins to a surface via a unique chemical linkage.

In collaboration with the Chen Lab, we were able to use SFG and ATR-FTIR to confirm that both immobilized constructs were oriented so that the active site faced the bulk solution. However, the immobilized V152C construct demonstrated a larger range of motion than the immobilized E147C. The thermal stability of the protein was determined by measuring the amount of activity of the immobilized enzyme after heating to a particular temperature and

cooling back to room temperature. It was shown that the immobilized V152C construct was less thermally stable than the immobilized E147C construct.

Along with the SFG and ATR-FTIR data, the thermal stability data suggests that a correlation may exist between the range of motion of an immobilized enzyme and thermal stability. To examine this possible correlation further, we collaborated with the Brooks lab to simulate the structure and dynamics of immobilized β -gal at different temperatures. Molecular dynamic simulations showed that immobilizing β -gal to the surface via the V152C construct increased the likelihood that the immobilized enzyme would make contact with the surface. Because a maleimide-derivatized surface is mildly hydrophobic, these protein-surface contacts often lead to interactions that disrupt the folding of the protein.

In chapter 4 we explored protein surface interactions more closely. We tethered the β -gal V152C to surfaces that vary in either hydrophobicity or electrostatic charge. Hydrophilic surfaces were shown to retain more activity for the immobilized β -gal V152C. For electrostatic charge, it was shown that a mixed charge surface allowed the protein to retain the highest level of activity.

In chapter 5, we explored the role of orientation on the activity of immobilized enzymes. Three β -gal constructs were used: a. the V152C construct described previously, the E227C construct that places the unique surface cysteine near to the active site, and the D308C construct, which also places the cysteine near the active site. When tethered to a surface, the active site of the V152C construct is oriented towards the bulk solution. For the immobilized E227C and D308C constructs, the active site is oriented towards the surface. There is little difference in the measured activity of the three immobilized constructs, suggesting that the substrate is still able to enter into the active site, either because it is significantly smaller than the clearance between the

active site and the surface, or because the movement of the protein exposes the active site for a sufficient amount of time.

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Chapter 2

Studying the Specific Activity and Orientation of Engineered β -Galactosidase Tethered to Self-Assembled Monolayers

2.1 Introduction

Some of the work described in this chapter has been published as, “Molecular Orientation of Enzymes Attached to Surfaces through Defined Chemical Linkages at the Solid–Liquid Interface” *J. Am. Chem. Soc.*, 2013, *135* (34), pp 12660–12669. Co-authors were very helpful in conducting this research. Yuwei Liu from the Chen Lab conducted and analyzed the SFG data.

Researchers have long observed that enzymes intentionally immobilized onto surfaces lose specific activity relative to the enzyme free in bulk solution. Whereas a considerable amount of research has been done on protein adsorption on surfaces, and how the electrostatic and hydrophobic interactions can influence the behavior of these immobilized enzymes, much less work has been done on studying protein-surface effects for enzymes covalently tethered to the surface. Even if the protein is attached to the surface via a single chemical linkage, the protein is still free to move, and non-covalent interactions with the surface are still possible. One can imagine that these interactions will have consequences for both activity and stability.

Another question that has not been studied in detail is the effect of enzyme orientation on the activity of tethered enzymes. Adsorbing and covalently attaching the enzyme randomly to the surface will place the active site in an orientation that may be less accommodating to the binding of substrate, and this, in turn, may have an effect on activity.

To explore these questions, we have chosen to study enzymes tethered to self-assembled monolayers (SAM). SAMs provide flat, chemically well-defined surfaces that can have customizable chemical features. One of two monolayers were used in this study, a maleimide-derivitized polyethylene glycol chain and octadecyl trichlorosilane (OTS). Polyethylene Glycol (PEG) chains will be tethered to glass surfaces via a triethylsilane moiety. PEG was chosen as the foundation for the monolayer because of its ability to resist non-specific protein adsorption. In addition, the solvent exposed end of the PEG monolayer can be derivitized with a variety of chemical groups, enabling us to create mixed monolayers with customizable charge or hydrophobicity. Maleimide-functionalized PEG chains was used to covalently attach the proteins to the surface via a unique surface cysteine. To compare the activity and orientation of covalently tethered vs. adsorbed protein, OTS monolayers were also used in this study. The hydrophobic nature of OTS promotes non-specific protein adsorption, which we hypothesize would lead to a more random surface tethering and decreased specific activity due to unfolding.

Site-directed mutagenesis was used to place these unique surface cysteine at various locations throughout the protein, allowing for control of orientation and attachment site. In collaboration with the Chen Lab, SFG and ATR-FTIR was used to determine the net enzyme orientation on the surface.

6-Phospho- β -galactosidase (β -Gal) from *L. lactis* was chosen as a model enzyme for this research because it is predicted to generate a large SFG signal due to its α -helical structure (on the basis of calculations using the computer software discussed above). Previous reports had demonstrated that the enzyme maintains activity when immobilized on various solid supports via physical adsorption, covalent binding, chemical aggregation, encapsulation, and entrapment to increase its stability and reusability.⁷¹⁻⁷⁷ The activity of β -Gal can be assayed using commercially available chromogenic or fluorogenic substrates, providing simple and sensitive ways to investigate the effects of immobilization on the activity. The enzyme adopts a β / α -barrel fold⁵⁸ (PDB entry 2PBG) with all of the α -helices pointing in approximately the same direction. This is important because it allows the orientation of the immobilized enzyme with respect to the surface to be experimentally determined by SFG.

Under relatively mild conditions (pH 6.5-7.5), maleimides will react specifically with reduced thiols to form a stable covalent linkage. This specificity is frequently used for site-specific bioconjugation (source) and more recently to tether biomolecules to surfaces (source). Because cysteines are not commonly found on the surface of proteins, a unique surface cysteine can be easily be mutated into the protein. For this project, β -gal engineered to have a single surface cysteine was tethered either covalently to the PEG monolayer via a maleimide moiety or adsorbed onto OTS. As a control, a “no-cysteine” β -gal construct was incubated with the SAM surface to measure potential non-specific protein adsorption.

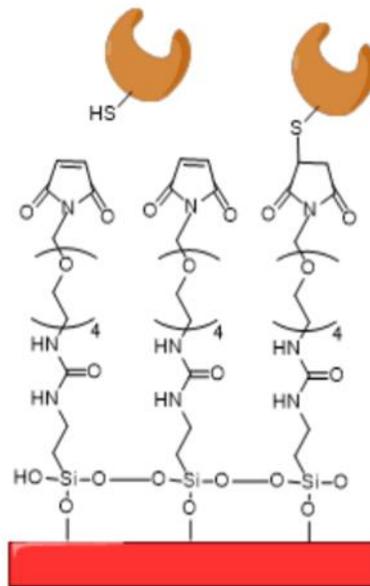


Figure 2.1: Tethering β -gal to PEG monolayers via a maleimide moiety.

2.2 Materials and Methods

2.2.1 β -Gal Constructs and Expression of Modified β -Gal

A synthetic gene, codon-optimized for expression in *E. coli*, encoding β -Gal (PDB 2PBG)⁵⁸ from *Lactococcus lactis* was obtained commercially (Genscript, New Jersey), subcloned into the expression vector pET28b so as to contain an N-terminal his-tag. The sequence was modified to replace all native cysteine residues with alanine. In addition, valine-152 was mutated to cysteine. A second construct, containing no cysteine, was made by using site-directed mutagenesis to mutate cysteine 152 back into a valine.

Expression vectors containing the β -Gal gene were transformed into *E. coli* BL21(DE3). Cells were grown in YT media containing 50 μ g/mL kanamycin to an OD of 0.6 at 600 nm.

Protein expression was induced by addition of 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG). The cell culture was harvested 4 h post induction by centrifugation at 5000 g at 4 °C for 20 min.

2.2.2 Purification of Recombinant β -Gal Cells (18 g damp weight) were resuspended in 90 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and a complete EDTA-free protease inhibitor cocktail tablet (Roche). Resuspended cells were sonicated using a 2 s on/8 s off pulse sequence for a total pulse time of 5 min. The lysate was centrifuged at 15000g at 4 °C for 20 min, and the supernatant from the lysate was incubated with 4 mL of Ni-NTA resin at 4 °C for 1 h. The Ni-NTA resin was then decanted into a chromatography column and washed with 50 mL of 20 mM imidazole dissolved in a 100 mM potassium phosphate buffer (pH 8) containing 300 mM NaCl, 10% glycerol, and 1 mM TCEP. β -Gal was eluted from the column using 10 mL of 200 mM imidazole dissolved in 100 mM potassium phosphate buffer (pH 8) containing 300 mM NaCl, 10% glycerol, and 1 mM TCEP. Fractions with pure enzyme were collected and dialyzed into 100 mM potassium phosphate buffer (pH 7.6) containing 10% glycerol and 1 mM TCEP. The enzyme was then concentrated using Amicon Ultra-15 centrifugal filters to a concentration of 50–100 μ M and stored frozen at -20 °C.

2.2.3 Enzyme Assay

The β -Gal activity was tested using either the fluorogenic substrate fluorescein β -digalactopyranoside (FDG) or the chromogenic substrate 2-nitrophenyl- β -galactopyranoside. The assay buffer typically contained 100 mM potassium phosphate (pH 7.6), 1 mM MgCl₂, 1 mM 2-mercaptoethanol, and 1% dimethyl sulfoxide (DMSO). For 2-nitrophenyl- β -galactopyranoside, the substrate concentrations varied between 0 and 1 mM, and the release of 2-nitrophenol was followed by the change in absorption at 412 nm. Assays using FDG were conducted at a concentration of 200 μ M; the excitation wavelength was 490 nm, and the emission spectra were scanned from 500 to 550 nm to detect release of fluorescein. When FDG was used as the substrate, the concentration of β -Gal was typically 10 nM; for assays using 2-nitrophenyl- β -galactopyranoside as the substrate, the enzyme concentration was 1 μ M. To determine the activity of β -Gal immobilized on glass beads, the assay was modified as follows. Glass beads loaded with 10 pmol of β Gal were added to a cuvette containing 990 μ L of 100 mM potassium phosphate buffer (pH 7.6) containing 1 mM MgCl. The reaction was started by adding 10 μ L of FDG in DMSO to final concentrations of 200 μ M FDG and 1% DMSO. The cuvette was shaken gently at room temperature to keep the beads suspended, and fluorescence measurements were taken discontinuously every 2 min for 30 min, allowing a short time for the beads to settle before the measurement was taken.

2.2.4 Free Thiol Determination

4 mg of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was dissolved in 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. 2.5 mL of buffer, 50 μ L of DTNB solution, and 250 μ L of enzyme solution were mixed together and incubated at room temperature for 10

min. The concentration of the free thiols was determined by measuring the absorbance at 412 nm.

2.2.5 Immobilization of β -Gal on glass beads

1 g of acid-washed glass beads (75 μ m average diameter) was incubated with anhydrous toluene containing 1 mM of either maleimide-EG4 (Mal-EG4) or octadecyl trichlorosilane (OTS) for 24 h at room temperature. Following incubation, the beads were washed with toluene, followed by methanol. After drying under vacuum, the beads were re-suspended in buffer containing 5 mM potassium phosphate, pH 7.2, 0.1 mM TCEP. A stock solution of β -Gal was added to the bead suspension to give a final enzyme concentration of 4 μ M. The reaction was incubated for 2 h at room temperature. The glass beads were then rinsed 3 times with 100 mM potassium phosphate buffer, pH 7.2 and used on the same day.

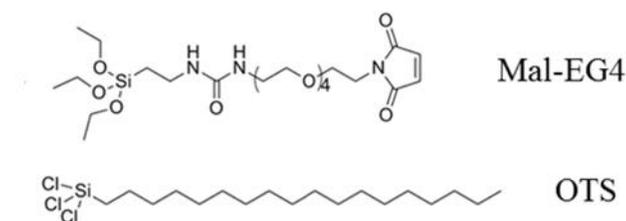


Figure 2.2: The two silane-derivatized molecules used to functionalize glass surfaces.

2.2.6 Determination of β -Gal concentrations

For determining enzyme concentrations in free solution, the absorbance at 280 nm was measured and concentrations calculated assuming $\epsilon = 110130 \text{ M}^{-1}\text{cm}^{-1}$ based on the amino acid composition of β -Gal. For enzymes immobilized on glass beads the amount of protein bound was determined using sodium bicinchoninate.¹ Reagent A was prepared by dissolving 8 g sodium carbonate monohydrate, 1.6 g sodium tartrate in water to a final volume of 100 mL, pH 11.25. Reagent B

was prepared by dissolving 4 g of sodium bicinchoninate in water to a final volume of 100 mL. Reagent C was prepared by dissolving 0.4 g of copper sulfate heptahydrate in water to a final volume of 10 mL. Reagents A, B, and C were mixed at a ratio of 25:25:1 (v/v/v) just before use. 50-100 mg of beads was mixed with 500 μ L Millipore water and 500 μ L of the reagent mixture. After vortexing, the mixture was incubated at 60 °C for 15 min. Following incubation, the mixture was allowed to cool to room temperature, before reading the UV absorbance at 562 nm.

2.2.7 Defining a Monolayer of Surface Tethered Proteins

Because we were interested in exploring protein/surface interactions directly, it is important to ensure that the immobilized proteins are in a monolayer. Multiple layers would suggest that some of the protein may be randomly adsorbed. To determine if the enzyme coverage of the surface is consistent with a monolayer, a suitable definition is necessary. As seen in Figure 3, it was assumed that β -gal is a globular protein with a diameter of 55Å, or 5.5nm. This would imply that a monolayer, on average, would contain no more than 2-3 molecules per 100 nm².

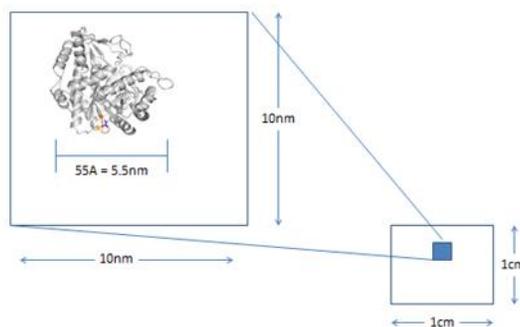


Figure 2.3: The size of β -Gal and the size of the monolayer

For a 1 cm² surface in a 1mL solution, the concentration of β -Gal is below the threshold necessary to measure activity. To increase the surface area per volume, glass beads were used. Each bead has an average diameter of 75 μ m. For a protein with a 5.5 nm diameter, the surfaces of these beads are effectively flat. Based on a bead diameter of 75 μ m, and assuming a footprint of \sim 100 nm² for β -Gal, monolayer coverage would result in a loading of \sim 0.27 pmol enzyme/mg of beads.

2.3 Results

2.3.1 Engineering S-Gal

6-phospho- β -galactoside (β -Gal) from *Lactobacillus lactis* serves as a useful model enzyme with which to examine the effects of surface attachment on activity and structure. It is a stable, monomeric enzyme for which the crystal structure has been determined and a simple and sensitive assay is available. Furthermore, the parallel orientation of the α -helices in the protein gives rise to a strong SFG signal for the backbone carbonyl groups, the polarization-dependent signal of which can be used to determine the orientation of the protein with respect to the surface normal.

Native β -Gal contains three cysteine residues, none of which are required for activity. In addition, the enzyme contains no disulfide bonds that could be required for structural stability. Therefore, a synthetic gene was constructed in which all three native cysteine residues were mutated to alanine. Next, to facilitate tethering of the enzyme to a maleimide-terminated SAM, Val-152 was mutated to cysteine. Residue 152 is on a surface loop (Figure 1C) and was chosen

to minimize any disruption of the secondary structure that covalent attachment of the enzyme to the surface may cause. Furthermore, it should orient the active site to face toward the bulk solvent, providing the substrate easy access to the active site.

A second construct containing no surface cysteine residues was also made. In this case, site directed mutagenesis was used on the original synthetic gene to revert Cys-152 back to a valine. Both the “no cysteine” and β -GalV152C enzymes were overexpressed and purified from *E. coli* by standard methods.

The kinetic parameters k_{cat} and K_m for both β -Gal enzymes, free in solution, were measured for the hydrolysis of the chromogenic substrate 2-nitrophenyl- β -galactopyranoside. For the β -Gal-V152C enzyme variant, $k_{cat} = 0.18 \pm 0.01 \text{ s}^{-1}$ and $K_m = 0.2 \pm 0.02 \text{ mM}$; for the “no cysteine” β -Gal enzyme variant, $k_{cat} = 0.35 \pm 0.03 \text{ s}^{-1}$ and $K_m = 0.1 \pm 0.01 \text{ mM}$. These values are similar to those reported for the wild-type enzyme, indicating that the engineered changes did not substantially affect the activity of the enzyme. The presence of a single reactive cysteine residue in β -Gal-V152C was confirmed using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent).

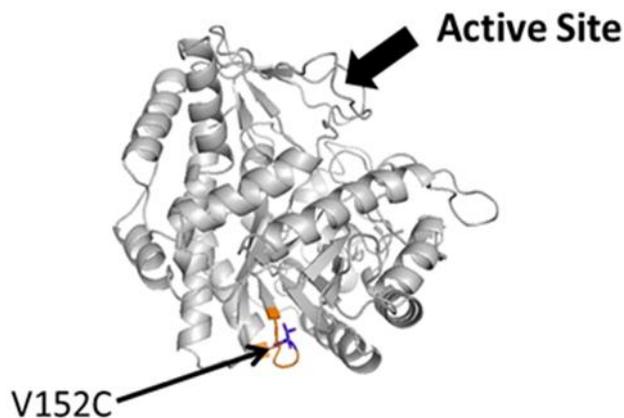


Figure 2.4: Crystal structure of β -Gal. Here the amino acid 152 was replaced by a cysteine group (β -Gal-V152C). The cysteine group can bind to the maleimide group on Mal-EG4 SAM to immobilize β -Gal to the surface. The binding site is opposite to the enzyme active site.

2.3.2 Measuring the Concentration of Surface Tethered Enzymes

MicroBCA is a well-established technique for determining the concentration of proteins in solution. Cu^{2+} will spontaneously chelate to protein and peptide backbones. Under basic conditions, these chelated Cu^{2+} will be reduced to Cu^+ and diffuse back to solution, where each Cu^+ will then chelate with bicinchoninic acid. The mechanism of the Cu reduction is not fully understood. The resulting bicinchoninic acid/ Cu^+ complex absorbs at 562nm. Because of the diffusion of the copper to and from the protein, we were able to adapt this assay to determine the concentration of proteins tethered to surfaces. Amino acid analysis was used to confirm the reliability of this assay.

The experimentally determined protein loading for β -Gal-V152C immobilized through Mal-EG4 linker was 0.14 pmol/mg of beads, suggesting that the specifically tethered enzyme is likely attached as a monolayer with few or no non-covalent interactions between enzyme molecules. For enzyme physically adsorbed on OTS-derivatized beads, the loading was 1.0 pmol/mg of beads, which is much greater than the concentration expected for a monolayer.

2.3.3 Activity of surface-immobilized β -Gal

Although β -Gal has favorable structural features for determining its surface orientation by SFG, the enzyme was not sufficiently active that we could directly measure β -Gal activity on the

prism surfaces used for SFG measurements. To enhance the sensitivity, we attached the enzyme to glass beads coated with either Mal-EG4 SAM or OTS SAM. On a microscopic level, the SAMs on the bead surface should be chemically identical to that of the SFG prisms, but the surface area is greatly increased. To increase sensitivity further, the fluorogenic substrate fluorescein-*-*galactopyranoside (FDG) was used to measure activity.

Using this substrate, the specific activity of β -Gal-V152C in free solution was $1.1 \text{ nmol min}^{-1}\text{mg}^{-1}$. The specific activity of β -Gal-V152C immobilized on Mal-EG4 derivatized glass beads was $1.08 \text{ nmol min}^{-1}\text{mg}^{-1}$ which is identical, within error, to the enzyme in free solution. In contrast the specific activity of β -Gal-V152C physically adsorbed on OTS-derivatized beads was $0.35 \text{ nmol min}^{-1}\text{mg}^{-1}$, which is only one third that of the enzyme in free solution.

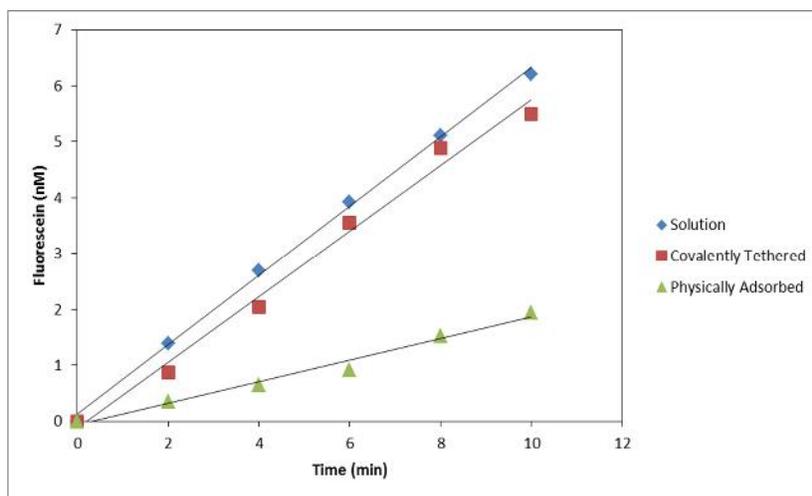
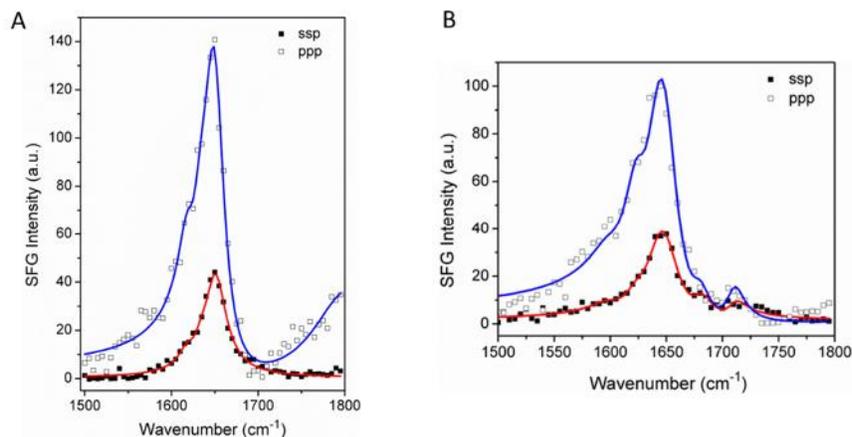


Figure 2.5: Measuring the amount of fluorescein produced per minute by β -gal from hydrolysis of the fluorogenic substrate fluorescein-*-*galactopyranoside. β -gal was assayed either free in solution (blue), covalently tethered to the surface via a unique surface cysteine (red) or physically adsorbed to the surface (green)

2.3.4 SFG and ATR-FTIR

In collaboration with the Chen lab, have used SFG and ATR-FTIR to measure the net orientation of the enzyme relative to the surface. As discussed in Chapter 1, polarized IR spectra are used to measure the net dipole of proteins immobilized to surfaces using polarized IR light.



For our studies, the IR spectra of the peptide amide-I, largely the result of backbone carbonyl stretches, was measured. Because backbone carbonyl groups are involved in secondary structure formation, the wavenumbers of these carbonyl groups change from α -helices to β -sheets to random coils, allowing the net dipole from specific secondary structural elements to be detected. Because of their parallel arrangement of carbonyl groups, α -helices provide a particularly strong signal that was exploited in these experiments.

Figure 2.6: SFG spectra for β -gal attached to a. Mal-PEG SAMs and b. OTS

It is interesting to observe stronger SFG signal intensity for β -Gal-V152C immobilized through Mal-EG4 linker compared to that physically adsorbed on OTS, as seen in Figure 6, even though the surface coverage of the latter is higher. SFG signal intensity is related to the surface coverage and orientation of functional groups or molecules (under the fixed visible and IR input

beam energies). For the chemical immobilization, the enzyme molecules more or less adopt a similar orientation (with the cysteine group facing the surface for immobilization), therefore the signal can be stronger. The signal should be proportional to the square of the surface coverage (assuming orientation is coverage independent, which is likely for chemical immobilization). For the physically adsorbed enzyme molecules, the orientation distribution can be much broader. Therefore SFG signals from enzyme molecules with different orientations can be canceled in some degrees. As a result, the detected SFG signal can be smaller even when the enzyme surface coverage is higher.

2.4 Discussion

In this work, specific immobilization of 6-phospho- β -galactosidase through a unique cysteinyl residue was achieved on SAMs containing maleimide end groups and oligo ethyleneglycol spacer segments. Based upon SFG studies done by the Chen Lab, the possible orientations of the immobilized β -Gal were determined to be in a region with tilt angles ranging from 15 to 30 and twist angles ranging from 60 to 130, as shown in Figure 7.

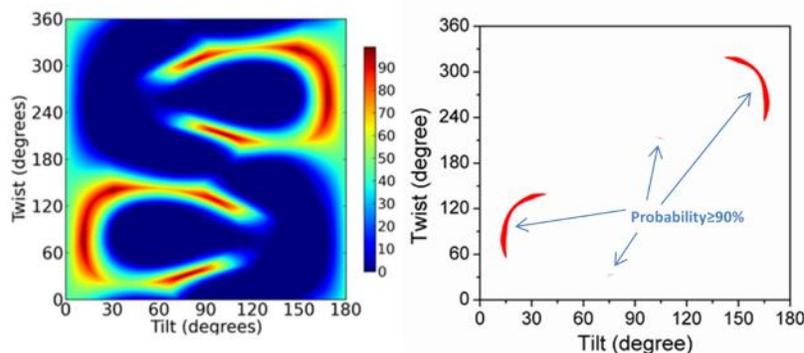


Figure 2.7. Possible orientation angle regions deduced based on both the SFG and ATR-FTIR measurements.

Colors indicate the quality of the match (100%=exact). The right figure plotted the possible orientation angles with probability $\geq 90\%$ in red.

On the other hand, 6-phospho- β -galactosidase non-specifically adsorbed onto hydrophobic octadecyl SAMs appears to be partially denatured and exhibits significantly reduced activity. As we discussed above, in our SFG and ATR-FTIR studies, we assume that the specifically immobilized enzymes do not significantly change their structures. Since the activity of the chemically immobilized β -Gal is similar to that in solution, and the enzyme orientation determined spectroscopically is reasonable, we believe that this assumption, at least in this case, is valid. In future experiments we aim to further characterize the possible conformational changes of surface tethered proteins, using isotope labeled proteins.

There are no published examples providing a detailed correlation between the directly measured orientation and specific activity of a surface-attached enzyme. Previous work in the field assumes a hypothetical orientation of the tethered enzyme based on the attachment site. This assumption, however, does not consider that dynamic movement of the enzyme and enzyme-surface interactions, may lead to a broad array of orientations, or to the enzyme assuming a completely different orientation than originally hypothesized. By correlating the measured orientation with retained activity, this work provides a systematic means to characterize interfacial orientation of immobilized enzymes. This is the first step in providing a more in-depth molecular characterization of protein-surface interactions that the field currently lacks.

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Chapter 3

The Role of Attachment Site on the Thermal Stability of Immobilized Enzymes

3.1 Introduction

The work in this chapter is the result of a collaboration. The coarse grain simulations of thermal stability were performed by Dr. Shuai Wei in the Brooks lab, and the SFG simulations were performed by Yuwei Liu of the Chen Lab.

The immobilization of enzymes on abiological surfaces plays a central role in a wide range of important technological applications, including industrial catalysis, drug delivery, medical diagnosis and biosensors.^(1, 2) Depending upon the application, immobilization may prolong the useful lifetime of the enzyme and/or facilitate its removal from the reaction and reuse. The attachment of enzymes to surfaces is known to significantly affect both enzyme activity and thermal stability, issues that play an important role determining the economic feasibility of using enzymes in biotechnological processes.⁽¹⁻⁴⁾

Despite these important applications, our understanding of how enzymes and proteins interact with abiological surfaces on the molecular level remains extremely limited. In part, this reflects the approaches that have been traditionally used to immobilize enzymes, which have relied on non-specific adsorption through electrostatic or hydrophobic interactions, or non-specific covalent cross-linking through the amino-groups of surface lysine residues.^(3, 5, 6) Such methods

are simple to employ, but result in poorly-defined, heterogeneous mixtures of proteins that are attached in different orientations and may be partly unfolded or constrained in inactive conformations or orientations. Such heterogeneous mixtures are ill-suited to detailed characterization of the interactions between the protein and surface substratum that have an important influence on protein structure, activity and stability.

In our studies we have used engineered enzymes that contain unique cysteinyl residues introduced at the desired attachment point on the protein's surface.^(7, 8) This allows the enzyme to be covalently attached to a suitable maleimide-functionalized surface, in a chemically well-defined manner. This approach yields a far more homogenous population of surface-tethered protein molecules, making it possible to examine how changing the tethering site alters the interaction between protein and surface and its effects on structure, activity and stability.

Here we report studies on 6-phospho- β -galactosidase (β -Gal) from *Lactobacillus lactis*,⁽⁹⁾ a representative of the class of glyco-hydrolases that have important uses in a variety of technological applications.⁽¹⁰⁾ We examined the properties of two enzyme variants tethered to a surface formed by an (ethylene glycol)₄-maleimide-terminated self-assembled monolayer (EG₄-maleimide SAM, Figure 1).

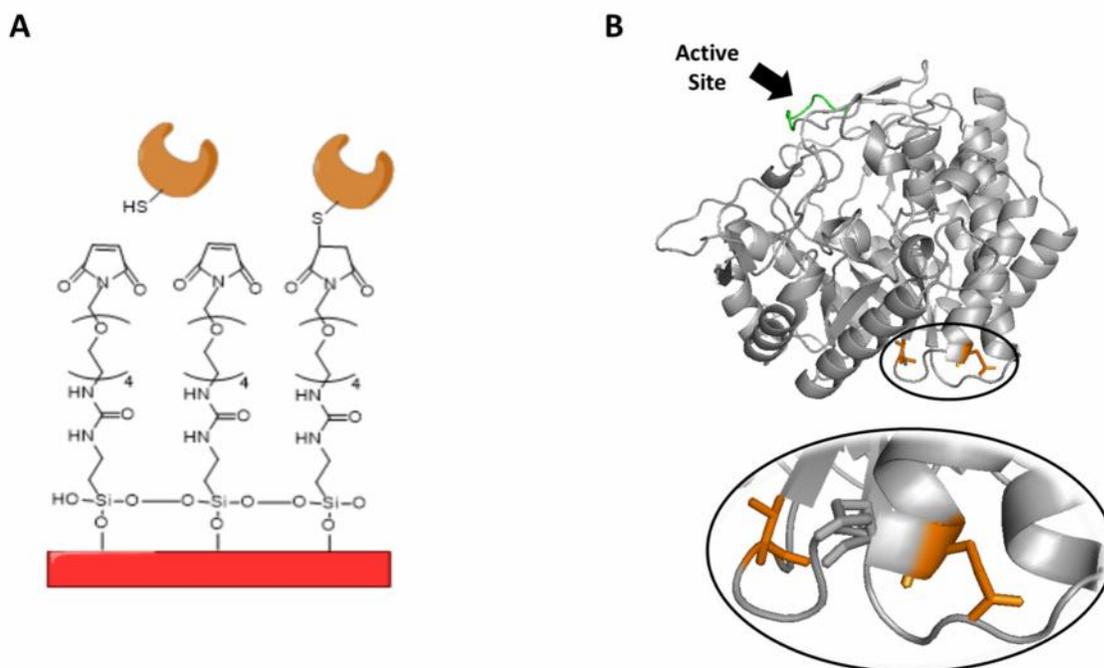


Figure 3.1 A) Structure of maleimide-terminated SAM on silica surface used in these studies to facilitate covalent attachment of proteins. B) Structure of β -gal, with active site loop indicated. The two surface residues, E147 (helix) and V152 (loop) that were mutated to cysteine to facilitate surface attachment are highlighted in gold.

In one case the enzyme was tethered through a flexible surface loop; in the other, through a more rigid α -helical element. The two immobilized enzymes have similar activities at room temperature, however their thermal stabilities differ significantly. Whereas the enzyme tethered at the α -helix position has thermal stability similar to β -Gal in solution, the thermal stability of loop-tethered enzyme is significantly lower. Coarse-grain molecular dynamics simulations of the surface-tethered enzymes were able to recapitulate the experimentally-determined thermal inactivation curves and facilitate a more detailed analysis of the orientation and fluctuations of the proteins as a complement to our spectroscopic analysis. Comparison of the unfolding trajectories for the tethered enzyme with the enzyme in free solution demonstrates the important role of

surface-protein interactions in the unfolded state. Further characterization of the enzymes using the surface-sensitive techniques sum frequency generation (SFG) spectroscopy and attenuated total reflectance (ATR) FT-IR spectroscopy⁽¹¹⁾ indicates that the loop-tethered enzyme and the helix-tethered enzyme have similar orientations at room temperature. However, for the loop-tethered enzyme the range of possible orientations deduced from spectroscopy is larger, indicating that it is likely more mobile and thus more likely to suffer surface-induced unfolding than the helix-tethered enzyme, in accord with the coarse grain simulations.

3.2 Materials and Methods

3.2.1 Design and Expression of Modified β -Galactosidase Constructs

The design of synthetic gene, codon-optimized for expression in *Escherichia coli*, encoding a variant of 6-phospho- β -galactoside (β -Gal) from *Lactobacillus lactis* (PDB entry 2PBG)⁽⁹⁾ in which all the native cysteine residues have been replaced by alanine, together with its expression and purification from *E. coli* BL21(DE3) cells have been described previously⁽¹²⁾. To introduce a unique cysteine into a surface exposed loop, Val-152 was mutated to cysteine by standard methods; similarly to introduce a unique cysteine into a surface exposed α -helix, Glu-147 was then mutated to cysteine. These proteins were expressed and purified in the same way as the “no cysteine” β -Gal variant⁽¹²⁾. Enzymes were stored at concentrations of 50–100 μ M at -80 °C.

3.2.2 Functionalization of Glass Beads for Enzyme Assay

75 μ M diameter acid-washed glass beads (Sigma) were incubated overnight in 1mM of maleimide-PEG₄–silane (Creative PEG Works, Winston, Salem, NC) in toluene. The beads were then washed with toluene followed by methanol, and 100 mM potassium phosphate, before being

vacuum dried. To attach enzymes, 200 mg aliquots of dry maleimide-functionalized beads were incubated in 1 mL of 100 mM potassium phosphate buffer, pH 7.6, containing 5 μ M of α -GalV152C or α -GalE147C respectively, pre-reduced with 1 mM TCEP, for 4 h at room temperature with gentle shaking. The beads were then washed with 3 x 1 mL of 100 mM potassium phosphate buffer and used immediately.

3.2.3 Enzyme Assay

Enzyme activity was determined using the fluorogenic substrate resorufin- α -galactopyranoside (Life Technologies, Grand Island, NY). All assays were performed in 1 mL of buffer containing 100 mM potassium phosphate, pH 7.6, 1 % DMSO, and 10 pmol of α -Gal. Assays were initiated by addition of resorufin- α -galactopyranoside to a final concentration of 50 μ M. Formation of resorufin was measured using fluorescence, with excitation at 571 nm and recording emission at 584 nm. For enzyme tethered to beads, 18 - 20 mg of beads were suspended in 1 mL of buffer in a 1.5 mL Eppendorf tube with shaking. After 1 min of shaking, the beads were allowed to settle before a 750 μ L aliquot was transferred to a cuvette and the fluorescence measured. The aliquot was then transferred back to the Eppendorf tube and shaken for a further 1 min and the process repeated. Typically 10 time points were recorded for each rate measurement.

To determine the kinetic parameters of the mutants, the chromogenic substrate 2-nitrophenyl- α -galactopyranoside (NPG) was used. The substrate concentrations varied between 0 and 1 mM, and the release of 2-nitrophenol was followed by the change in absorption at 412 nm. For assays using 2-nitrophenyl- α -galactopyranoside as the substrate, the enzyme concentration was 1 μ M.

3.2.4 Thermal Stability of β -Gal

To examine the thermal stability of β -Gal variants in free solution, 100 μ L aliquots of a solution containing 100 nM β -gal in 100 mM potassium phosphate buffer, pH 7.6 were heated at temperatures ranging from 25 - 60 $^{\circ}$ C for 10 minutes using a thermocycler, followed by a rapid cooling to room temperature. The enzyme solution was diluted to 10 nM prior to the assaying for residue enzyme activity at 25 - 60 $^{\circ}$ C.

To examine the thermal stability of β -gal variants tethered to glass beads, 18 – 20 mg aliquots of enzyme-functionalized beads, corresponding to 10 pmol of enzyme, were suspended in 100 μ L of 100 mM potassium phosphate pH 7.6 and were heated and cooled as described. The bead suspension was diluted with 900 μ L of room temperature buffer was added prior to assay for residual enzyme activity. To estimate T_m , and the slope of the curve at T_m , thermal stability data were fit to equation 1 as described previously^(13, 14)

$$y = A_2 + \frac{A_1 - A_2}{1 + \exp\left(\frac{T_m - T}{\text{slope}}\right)}$$

Equation 1

Where A_1 and A_2 are the upper and lower asymptotes, respectively, of the enzyme activity and T_m is the temperature at which 50% of the initial activity remains.

3.2.5 Sample preparation for SFG and ATR FTIR vibrational spectroscopic analyses

Right-angle CaF₂ prisms (Altos Photonics, Bozeman, MT, USA) coated with 100 nm SiO₂ were reacted with 1 mM maleimide-EG₄-silane in anhydrous toluene for 24 h at room temperature to produce EG₄-maleimide-terminated SAM surface for protein attachment as described previously.

SFG theory and applications have been extensively published before^(8, 11, 15, 16) and are therefore not detailed here. SFG spectra were recorded on a custom made apparatus purchased from EKSPILA, Vilnius, Lithuania; details of the experimental setup has been described previously⁽¹²⁾. In this chapter, near-total-reflection geometry was used with the EG₄-maleimide functionalized right-angle CaF₂ prisms. Proteins were covalently attached to the prisms by immersing the surface in a 2 mL reservoir containing 5 mM pH 7.2 phosphate buffer (PB) and 0.1 mM TCEP. The appropriate volume of an enzyme stock solution, pre-reduced with 1 mM TCEP at room temperature for 2 h to reduce any potential disulfide bonds, was added to the reservoir to a final concentration of 4 μM. After the system was equilibrated, SFG spectra with a polarization combination of ssp (s-polarized sum frequency output, s-polarized visible input, and p-polarized infrared input) and ppp were collected and used for orientation analysis as described previously.^(12, 17) All SFG spectra were normalized according to the intensities of the input IR and visible beams.

ATR-FTIR spectra were recorded using a Nicolet Magna 550 FTIR spectrometer. Experiments were carried out using ZnSe total internal reflection crystal (Crystran Ltd. Dorset, England) deposited with a 50 nm layer of SiO₂ and functionalized with EG₄-maleimide SAM as described previously. 1.6 mL of 5 mM phosphate buffer, pD 7.2, containing 0.1 mM TCEP in D₂O was added to the trough above the SAM-functionalized crystal; D₂O was used to avoid possible signal confusion between the O-H bending mode and the peptide amide I mode and to ensure a better S/N ratio in the peptide amide I band region. After recording background spectra,

the appropriate volume of an enzyme stock solution, pre-reduced with 1 mM TCEP at room temperature for 2 h to reduce any potential disulfide bonds, was added to the reservoir to a final concentration of 4 μ M and allowed to react with the surface. The s- and p-polarized ATR-FTIR spectra of the enzyme covalently tethered to the SAM were recorded after the system reached equilibrium and used for orientation analysis as described previously.^(12, 18)

3.2.6 Coarse Grain Molecular Dynamics Simulations

Enzyme simulations used the Karanicolas and Brooks' structure centric (Go) protein-model^(19, 20). This model describes each residue by one site placed at the C position of the residue. Native contacts are defined in this model based on the hydrogen bonding between backbone atoms or side chain/ side chain interactions.⁽¹⁹⁾ It has been shown that, using this model, the protein folding free energy surface and the folding mechanisms are consistently reproduced.⁽¹⁹⁻²²⁾

The simulations described here used a recently developed coarse grain model of protein-surface interactions⁽²³⁾ based on and incorporating the force field of Karanicolas and Brooks.^(19, 20) The potential function is represented by equation 2, in which the first three terms of the potential function between the protein and the surface successfully capture the adsorption well and the energy barrier features as observed in many experimental works^(24, 25). Furthermore, the two third power terms were added to the function to account for hydrophobic effects of different Self Assembled Monolayer (SAM) surfaces and different residues in a protein or peptide by using the hydrophobic index of the surfaces χ_s and amino acids χ_p . The five-term model was well parameterized and parameters used in this chapter, are the same as previously published⁽²³⁾.

$$V_{surface} = \sum_i^N \left\{ \pi \rho \sigma_i^3 \epsilon_i \left[\theta_1 \left(\frac{\sigma_i}{z_{is}} \right)^9 - \theta_2 \left(\frac{\sigma_i}{z_{is}} \right)^7 + \theta_3 \left(\frac{\sigma_i}{z_{is}} \right)^3 - (\theta_s \chi_s - 4.5) + \theta_p \chi_p \right] \left(\frac{\sigma_i}{z_{is}} \right)^3 \right\}$$

Equation 2

The hydrophobicity of the maleimide SAM surface, which is the SAM surface examined in this work, is set to be moderately hydrophilic with a χ_s of 1.5 to represent the measured contact angle of 65° from previous experimental work⁽²⁶⁾. The bond between the maleimide surface and the cysteine thiol is simulated with a harmonic restraint with an interaction potential of the form:

$$U_{restraint} = \frac{1}{2} k_r (r - r_{eq})^2 \quad \text{Equation 3}$$

where $k_r = 10$ kcal/mol is the parameter describing the strength of the tethering restraint, r is the distance of the tethering site from the origin of the surface (0, 0, 0), and $r_{eq} = 5.8$ Å is the equilibrium distance from the tethering site to the surface origin. The tethering length of 5.8 Å approximates the distance between the maleimide surface and the C of the cysteine residue at the tethering site.

-Gal was simulated using the Go-like model described above starting with the previously determined x-ray structure PDB ID 2PBG. -Gal was tethered to the moderately-hydrophilic surface with a cysteine mutated at either residue 147 or residue 152. Both locations for the cysteine residue, when tethered to the maleimide monolayer, orient the active site towards the bulk solution.

To obtain protein thermal stability data in the bulk and on the maleimide surface with different tethering sites, multiple folded and unfolded samplings are required in the simulation. This is achieved by using the replica exchange molecular dynamics (REMD)

method.⁽²⁷⁻³⁰⁾ In this work, 24 replicas with different temperatures in the range of 270 K to 360 K (as listed in Table S1) are used in all three cases (in the bulk and on the surface with site 147 and site 152). Swaps were attempted every 2000 steps, and temperature increments between adjacent replicas ranged from 2.5 to 10 degrees. The smaller increments were used close to the melting temperature and the larger increments further away. The canonical ensemble was used for each replica, and the temperature was maintained by the Nosé-Hoover-Chain integration method with 3 thermostates of mass 10^{-26} kg $^{\circ}$ A² (31-33). Each simulation was performed with 10 million steps of equilibrium and 30 million steps of production with the time step of 1 fs/step. A small step size is used to avoid residues from moving beyond the surface.

Table S1 Temperatures of replicas as in the REMD simulation

Replica	1	2	3	4	5	6	7	8	9	10	11	12
T (K)	270	280	285	290	295	300	302.5	305	307.5	310	312.5	315
Replica	13	14	15	16	17	18	19	20	21	22	23	24
T (K)	317.5	320	322.5	325	327.5	330	332.5	335	337.5	340	350	360

3.2.7 Calculation of Thermodynamic Quantities

The metrics used to quantify stability were calculated from simulation data using standard methods from statistical mechanics. The melting point, T_m , is determined as the temperature at which only 50% native contacts are present, which will be shown as the transition point of the fractional nativeness curve. The instantaneous fractional nativeness, Q , is the ratio of the number of native contacts formed at a particular instance relative to the total number of native contacts possible. From the simulations, the average of the fractional

nativeness, Q , can be calculated using equation 4:

$$Q(T) = \langle Q \rangle_T = \frac{\sum_U Q(U)\Omega(U)e^{-\beta U}}{\sum_U \Omega(U)e^{-\beta U}} \quad \text{Equation 4}$$

where $\beta = \frac{1}{k_B T}$, and k_B is Boltzmann's constant and T is the temperature. U is the potential energy, and the $\langle \rangle$'s denote the average of the corresponding quantities. The key quantity needed to evaluate Equation 3 is the density of states, $\Omega(U)$, which is calculated using the Weighted Histogram Analysis Method (WHAM)⁽³⁴⁾ from the data obtained from the replica exchange simulations.

3.3 Results

3.3.1 Expression, Purification, and Characterization of β -Gal Constructs

β -phospho- β -galactoside (β -Gal) from *Lactobacillus lactis* serves as a useful model enzyme with which to examine the effects of surface attachment on activity and structure. It is a stable, monomeric enzyme for which the crystal structure has been determined and a simple and sensitive assay is available^(9,35). Furthermore, the parallel orientation of the α -helices in the protein gives rise to a strong SFG signal for the backbone carbonyl groups, the polarization-dependent signal of which can be used to determine the orientation of the protein with respect to the surface normal.^(11, 36, 37) We have previously validated this approach using the β -Gal V152C variant covalently tethered to an EG₄-maleimide-functionalized silica surface.⁽⁸⁾ Our results indicated that the tethered enzyme retains a significant fraction of its activity and its spectroscopically-deduced orientation with respect to the surface is consistent with its attachment point

We wanted to compare the effects on structure, activity and stability of tethering an enzyme through a flexible element such as a loop with tethering through a rigid structural element, such as an α -helix. This could potentially alter activity through non-covalent interactions between the enzyme and surface or possibly affect large scale, low frequency vibrational modes that have been shown to be important for catalysis in a number of enzymes.^(38, 39) A previously described variant, β -Gal V152C, allowed the enzyme to be tethered through a flexible loop and by introducing a cysteine residue at Glu-147 (β -Gal E147C variant) we facilitated attachment at an adjacent surface-exposed α -helical position (Figure 1). The close proximity of these two attachment points was intended to preserve the orientation of the enzyme with respect to the surface. Steady state kinetic analysis of these β -Gal variants in free solution, using 2-nitrophenol-galactose as a substrate,⁽¹²⁾ indicated that the introduction of cysteine at these positions has no effect on enzyme activity. For β -Gal V152C, $k_{\text{cat}} = 0.18 \pm 0.02 \text{ s}^{-1}$ and $K_{\text{M}} = 0.17 \pm 0.02 \text{ mM}$; for β -Gal E147C, $k_{\text{cat}} = 0.16 \pm 0.02 \text{ s}^{-1}$ and $K_{\text{M}} = 0.21 \pm 0.02 \text{ mM}$. These values are within error of the values for the wild type enzyme⁽³⁵⁾ and a previously engineered “no cysteine” variant.⁽¹²⁾

3.3.2 Immobilization of β -Gal to Maleimide-Terminated Self Assembled Monolayers

Both β -Gal variants were covalently coupled through their respective cysteinyl residues to 75 micron glass beads that had been functionalized with EG₄-maleimide-terminated SAM (Figure 1A). The variants coupled with similar efficiency; typically 0.03 μg protein/mg of beads were incorporated, corresponding to an approximate surface coverage of 2 molecules of β -Gal per 100 nm^2 . This value is similar to that expected for a monolayer of protein, assuming the beads to be uniform spheres and the glass surface to be atomically flat.

The specific activity of the surface immobilized enzymes was determined using the more sensitive fluorogenic substrate resorufin- β -galactopyranoside (rbg). In solution, the β -Gal variants exhibited similar specific activities: 2.7 ± 0.2 nmol/min/mg protein for β -Gal V152C and 2.6 ± 0.3 nmol/min/mg protein for β -Gal E147C. Upon coupling to EG₄-maleimide-SAM, the specific activity of the enzymes decreased slightly: 2.0 nmol/min/mg protein for surface tethered β -Gal V152C and 1.8 nmol/min/mg protein for surface tethered β -Gal E147C.

3.3.3 Thermal Stability of Immobilized Enzymes

Immobilized enzymes often display quite different thermal stabilities to those in free solution. To determine how tethering β -Gal to the SAM surface affected the thermal stability, aliquots of enzyme, either free in solution or covalently tethered to glass beads, were heated at temperatures ranging from 24 – 60° C for 10 min and then cooled to room temperature using a thermocycler. The enzymatic activity remaining was measured and normalized to activity at 25°C. Under these conditions thermal unfolding of β -Gal is irreversible and the fraction of activity remaining reflects the population of enzymes that remain folded at a given temperature.

In solution both β -Gal-V152C and β -Gal-E147C exhibit sharp thermal unfolding curves that are identical within error (Figure 2).

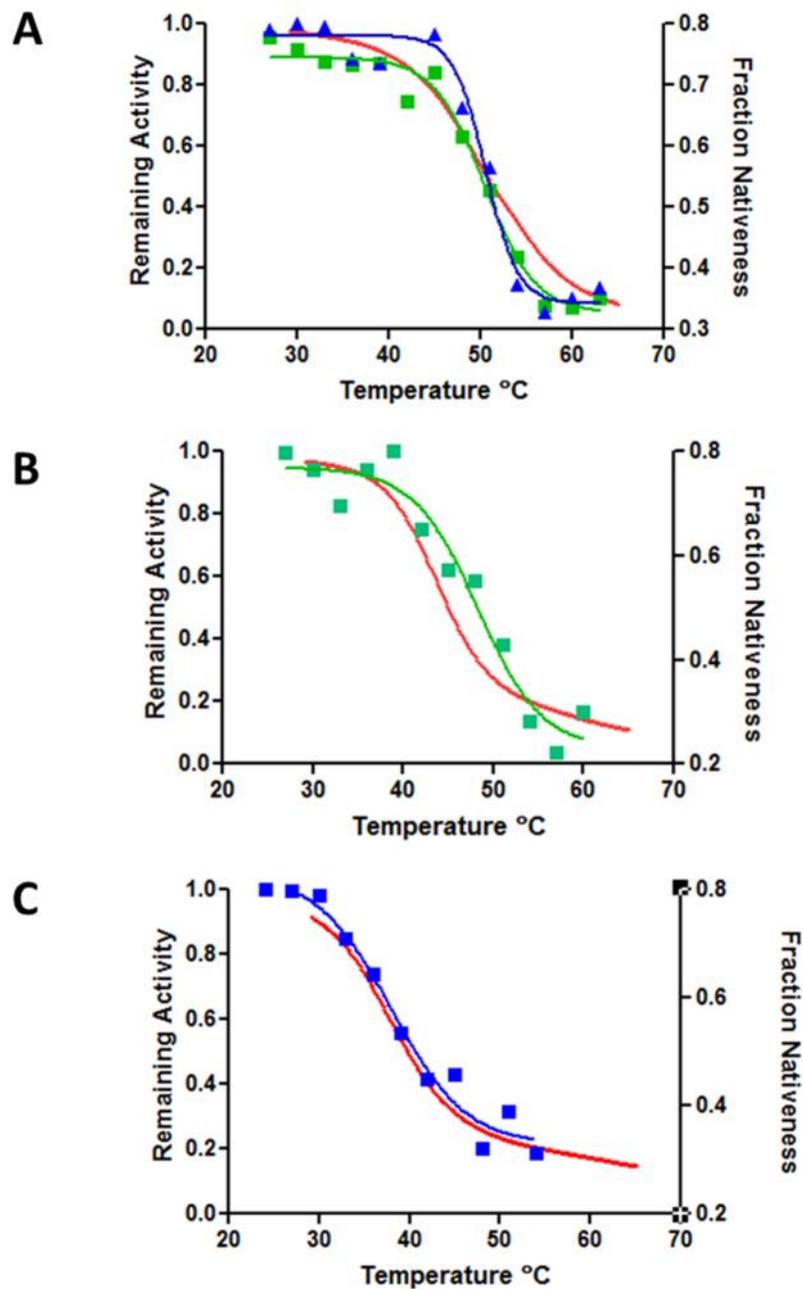


Figure 3.2: Comparison of experimental and computationally determined thermal stability for β -gal in solution and tethered to EG₄-maleimide SAM surface. **A)** Thermal stability curves (fractional activity remaining after heating at given temperature for 10 min) for β -Gal V152C (blue triangles) and β -Gal E147C (green squares) in free solution. **B)** Thermal stability curve for β -Gal E147C tethered to EG₄-maleimide SAM surface (green squares). **C)** Thermal stability curve for β -Gal V152C tethered to EG₄-maleimide SAM surface (blue squares). In each panel the red line is the computationally determined thermal unfolding curve (fraction nativeness).

Unexpectedly, the surface immobilized enzymes differed significantly in their thermal stabilities. Whereas the β -Gal-E147C variant, exhibited only a small decrease in $T_{1/2}$ from 50.5 °C in solution to 48 °C on the surface, for β -Gal-V152C $T_{1/2}$ decreased substantially from 50.6 °C in solution to 38 °C on the surface and the thermal unfolding curve was significantly broadened, as measured by the slope at $T_{1/2}$. These data, summarized in Table 1, suggest that interactions of the enzyme with the maleimide-terminated SAM surface that depend upon the specific site of covalent attachment may be responsible for the observed changes in thermal stability.

	$T_{1/2}$ (expt, °C)	T_m (calc, °C)	$T_{1/2} - T_m$ (°C)	$\Delta\Delta G_{\text{fold}}$ (calc relative to solution, KJmol^{-1})
β -Gal (solution)	51	48	3	0.0
β -Gal E147C	48	40	8	4.6
β -Gal V152C	38	34	4	5.9

Table 1 Comparison of experimental $T_{1/2}$ values and calculated T_m values for thermal unfolding of β -Gal in free solution and tethered to EG₄-maleimide terminated SAM surface

Using coarse grain simulations, Dr. Shuai Wei of the Brooks Lab simulated the thermal melt curves for β -Gal tethered to a mildly hydrophobic surface. The number of hydrogen bonds within the structure were analyzed and compared to the number found in the published crystal

structure (PDB#: 2PBG). This percentage was used to estimate the percentage of protein that remains folded. As shown in Figure 2, these curves are in good agreement with the experimental data. Additionally, Figure 3 below shows the simulations for the two immobilized constructs, V152C and E147C. The V172C construct begins to unfold while the E147C still maintains structural integrity. In addition, it is clear from the images that the unfolding of the immobilized enzymes are driven by protein-surface interactions.

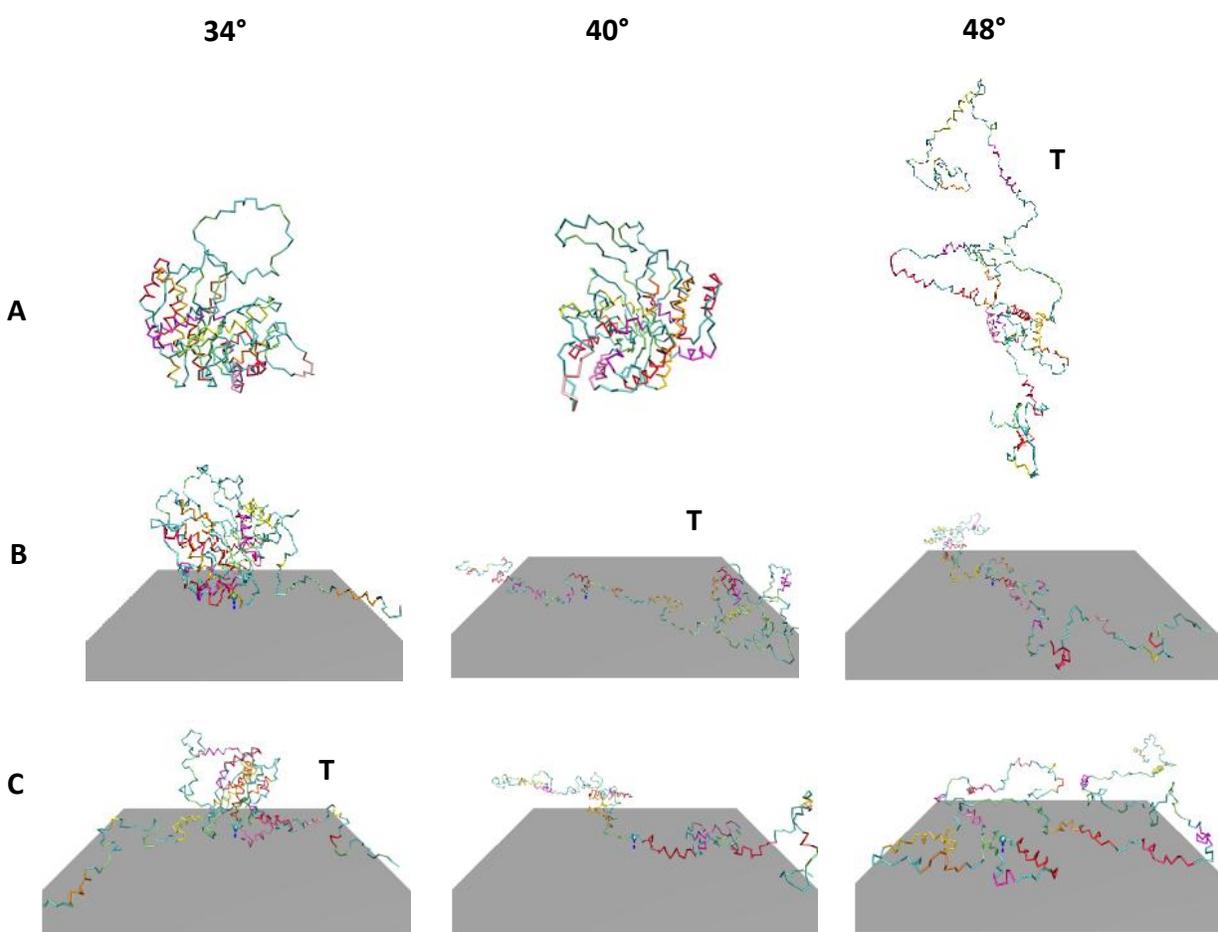


Figure 3.3: Coarse grain simulations of thermal unfolding of β -Gal. Representative structures are shown after 3 ns of simulation at 3 different temperatures corresponding to the T_m of each enzyme form; warm colors represent α -helices, cool colors represent β -sheets and loop regions. **A)** β -Gal in free solution; **B)** β -Gal tethered to the surface

through position 147 (helix); C) β -Gal tethered to the surface through position 152 (loop). A snapshot of one structure corresponding the T_m of each enzyme form is indicated. The attachment point for each surface-tethered enzyme is indicated by an arrow.

3.3.4 Orientation of Tethered Enzymes with Respect to Surface

As described in Chapter 2, SFG studies were carried out by Yuwei Liu of the Chen Lab to determine the orientation of both the V152C and the E147C constructs when tethered to the maleimide-terminated SAM. The results of this study are shown in Figure 4:

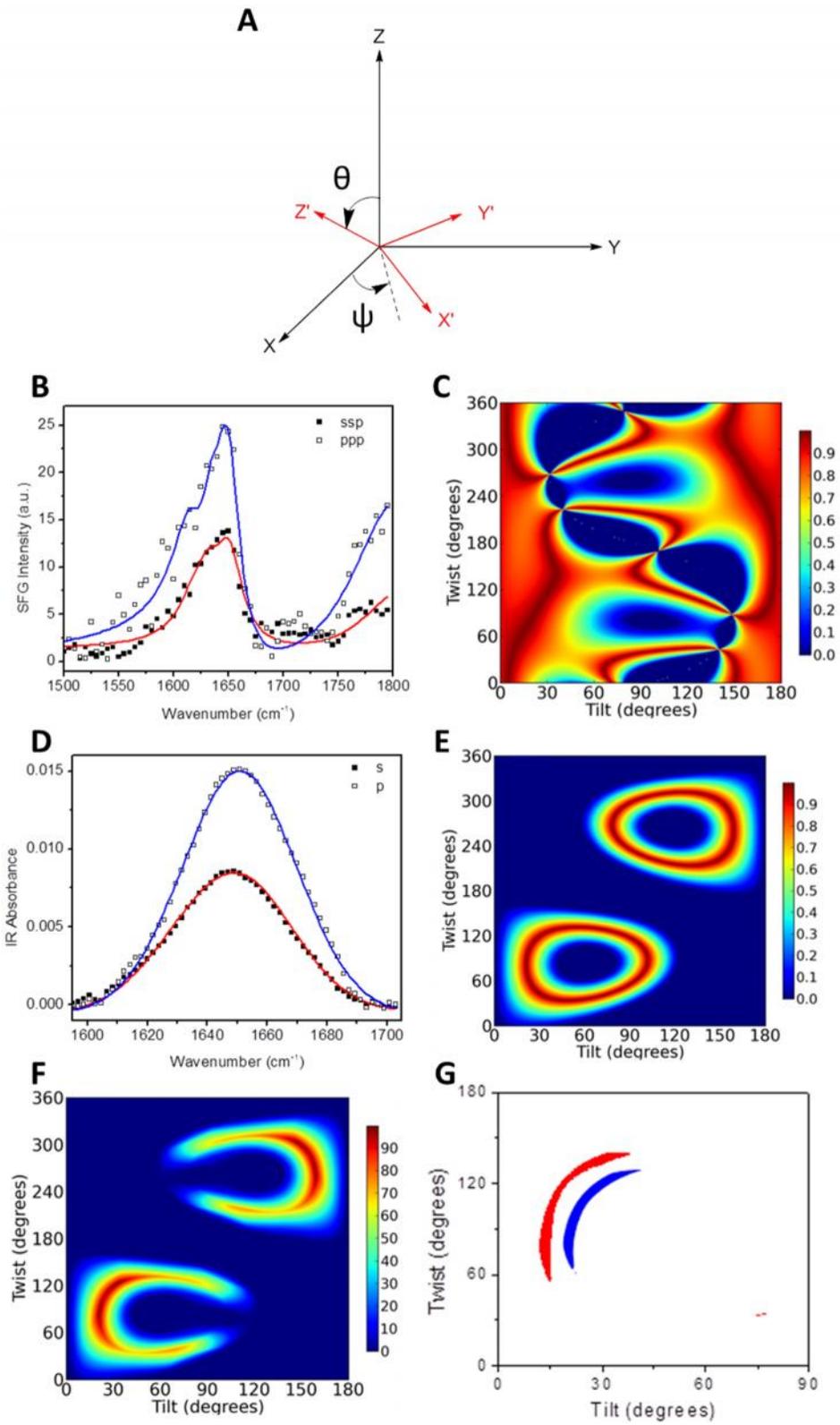


Figure 4.4 Determination of surface orientation of β -Gal-E147C. **A)** Definition of twist (θ) and tilt (ϕ) angles using an Euler rotation. **B)** SFG spectra of β -Gal-E147C tethered to EG4-maleimide SAM on silica substrate. **C)** Heat map showing possible orientation angle regions deduced from spectra in panel B. **D)** ATR-FTIR spectra of β -Gal-E147C tethered to EG4-maleimide SAM on silica substrate. **E)** Heat map showing possible orientation angle regions deduced from spectra in panel D. **F)** Heat map showing possible orientation angle regions consistent both the SFG and ATR-FTIR measurements. Colors indicate the quality of the match. **G)** Plots of possible orientation angles with probability $\geq 90\%$ β -Gal-E147C in blue and comparison with previously determined data²⁶ for β -Gal-V152C in red.

We compared the possible range of orientations deduced for β -Gal-E147C with those deduced for β -Gal-V152C⁽¹²⁾ (Figure 4G). (In Chapter 2, we discussed the orientation of the β -Gal-V152C variant,⁽¹²⁾ and showed that the deduced range of tilt and twist angles relative to the surface normal is consistent with its attachment through the loop residue 152.) As expected, the orientation angles are similar for the two constructs, however, the possible combinations of twist and tilt angles for β -Gal-V152C span a wider range than those for β -Gal-E147C. In addition, there is a small possible orientation area with large tilt angles for β -Gal-V152C (Figure 4G). This difference is consistent with residue 152 residing on a flexible loop that allows a great freedom of orientation, whereas the more rigid, helical location may place more constraints on the orientation of the enzyme. This also suggests that the β -Gal-V152C-tethered enzyme is more likely to come into contact with the surface through a large excursion in tilt angle than is the enzyme tethered through residue 147. As we discuss below, such protein-surface interactions are likely to be destabilizing.

3.4 Discussion

The immobilization of proteins on abiotic surfaces is important in a wide range of applications including medical implants, drug delivery, sensors and diagnostic testing, bioseparation technologies and bioreactors^(1, 3, 6, 40). Whereas it is well established that the interaction of proteins with surfaces have a profound influence on their structure and activity, we currently lack the detailed understanding of these interactions necessary to engineer immobilized proteins for optimal performance. To dissect this complex problem, we have used chemically well-defined SAM as surfaces and engineered enzymes to allow their attachment at precisely defined positions on the protein surface. This results in a population of surface-immobilized enzymes that are sufficiently uniform to permit detailed characterization by spectroscopic and computational methods.

We initially chose positions 147 and 152 in β -Gal as surface attachment points to explore the effect of secondary structural context – rigid helix versus flexible loop – on enzyme activity. We reasoned that the choice of attach point might affect large scale, low frequency vibrational modes that have been shown to be important for catalysis in a number of enzymes.^(38, 39) In practice, the immobilized enzymes were found to possess very similar specific activities, suggesting that the precise position of the tethering point is not critical for activity. Both of the tethered enzymes adopt a similar range of orientations, consistent with the position of the tethering points, as determined by analysis of their SFG and ATR-FTIR vibrational spectra (Figure 4) and simulations.

Given the similarity of the two surface tethered enzymes, we were surprised to find a marked difference in their thermal stabilities. However, valuable insights into the origin of these differences have been provided by coarse grain simulations that replicate with reasonable accuracy the experimentally determined thermal stability curves (Figure 2). Attachment through a flexible loop appears to provide more opportunity for hydrophobic residues, transiently exposed during localized unfolding of the protein, to form favorable interactions with the surface. Moreover, this interpretation is supported by spectroscopic analyses that indicate the loop-tethered enzyme samples a wider range of tilt angles than the enzyme attached through the adjacent helix.

Although the SAM surfaces employed in these studies are less complex than the solid supports, e.g. polystyrene beads, commonly used in biotechnological applications, we believe these results do have significant implications for the rational design of solid-phase supported biocatalysts. First, they demonstrate the importance of the choice of attachment point on the stability of the immobilized enzyme. Furthermore, they imply that the non-specific covalent cross-linking and non-covalent physisorption methods commonly used to immobilize enzymes likely result in biomaterials that are sub-optimal with respect to their activity and thermal stability.

Our results suggest that by better understanding the interactions between enzyme and surface it should be possible to design catalysts with enhanced thermal stability and improved activity, and potentially expand the range of enzymes that can be used in industrial processes. In particular, the use of computational methods to systematically screen multiple attachment sites on an enzyme of interest *in silico*, and thereby identify those that are likely to result in biomaterials with high thermal stabilities, appears a promising avenue for optimizing surface-supported enzyme catalysts.

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Chapter 4

The Effect of Surface Hydrophobicity and Electrostatic Charge on the Activity and Stability of Surface Immobilized β -Galactosidase

4.1 Introduction

As with the other chapters, the work in this chapter is the result of a collaboration. The coarse grain simulations of thermal stability were performed by Dr. Shuai Wei in the Brooks lab.

It has been long observed that the activity of an enzyme decreases when tethered to a surface. As discussed in Chapter 1, there are a variety of hypotheses for this decrease, involving hydrophobic to electrostatic interactions that result in protein-surface interactions that destabilize the native structure of the enzyme.

In addition, the stability of an enzyme is also affected. While some researchers claim that stability is increased with immobilization others have shown that stability can decrease¹. In Chapter 3, we showed that tethering a β -gal to a maleimide-terminated surface decreased the thermal stability. This decrease was shown to be the result of protein-surface interactions. Tethering β -gal to the surface via a flexible loop decreased the thermal stability to a greater degree than tethering to a rigid helix. It was shown through coarse grain simulations that this difference was the result of increased interaction of β -gal with the surface when tethered to the loop.

Most of the studies on protein-surface interactions concern random physical adsorption. Much less work has been done to study proteins covalently tethered to surfaces. Early work in

the field was largely concentrated on simulations¹. Researchers showed that any gains in stability of the immobilized enzyme arose from entropic effects – the existence of the surface restricted the volume within which the immobilized enzyme could unfold. A central assumption in these simulations is that the surface is neutral and does not interact with the protein. Therefore, the surface was simulated to have no lasting interactions with the immobilized enzyme.

As discussed in Chapter 1, surfaces are rarely neutral to protein interactions. While it is expected that hydrophobic and charged surfaces will interact with proteins, the work of George Whiteside² showed that terminal hydroxyl groups also can promote interactions through hydrogen bonding with the bound protein. These interactions will presumably also have an effect on thermal stability.

Self-Assembled Monolayers present an opportunity to explore these protein-surface interactions in more detail. Unlike adsorption, where the surface must exhibit a net attractive interaction with the protein, for covalent attachment, the surface can be either repulsion or attraction. Chemical moieties designed to covalently react with the protein can be mixed with other moieties with varying electrostatic and hydrophobic properties.

As discussed in Chapter 3, a fully maleimide-terminated surface decreases the thermal stability of immobilized α -gal relative to α -gal free in solution. Since it was shown that this decrease was due to protein-surface interactions, we extended these studies to include surfaces with a variety of hydrophobic and electrostatic properties. We examined the role of maleimide density by spacing maleimide derivatized PEG linkers with ones derivatized with OH. After finding an optimal maleimide density, we then explored the effects of hydrophobicity by changing the ratio of hydroxyl terminated linkers methyl terminated linkers. Finally, we

explored surface charge by changing the ratio of amine terminated linkers with those terminated with a carboxylic acid.

4.2 Materials and Methods

4.2.1 Materials

75 μ m acid washed glass beads were purchased from Sigma Aldrich. Alkyne triethylsilane was purchased from Gelest. Azido-PEG₃-Maleimide was purchased from Click Chemistry Tools. Azido-PEG₃-OH and Azido-PEG₃-COOH were purchased from Conju-Probe. Resorufin- β -galactopyranoside was obtained from Life Technologies (Grand Island, NY).

4.2.2 Functionalizing Glass Beads with Self Assembled Monolayers

Acid washed glass beads were shaken overnight at room temperature with 1mM of alkyne triethylsilane in anhydrous toluene. After washing three times with toluene to remove excess alkyne triethylsilane, the surface was shaken overnight at room temperature with 10mM of azido- Azido-PEG₃ – X, where X could be maleimide, hydroxyl, methyl, amide, or carboxylic acid. Excess Azido-PEG₃ – X was washed 3x to remove excess linker. The beads were then dried using a vacuum and stored in a desiccator at 4°C until use.

4.2.3 Functionalizing Protein to Maleimide

Stock solutions of β -gal were incubated with 1mM of TCEP for 20 minutes to reduce any disulfide bonds that may have formed. Maleimide derivatized beads were incubated with 5 μ M

of reduced β -gal for 4 hours at room temperature with gentle shaking. Excess protein was washed 3x with buffer + 2% Tween20 following a wash 3x with buffer. Protein functionalized beads were used immediately.

4.2.4 Assaying the Activity of Surface Tethered β -Gal

Enzyme activity was determined using the fluorogenic substrate resorufin- β -galactopyranoside (Life Technologies, Grand Island, NY). All assays were performed in 1 mL of buffer containing 100 mM potassium phosphate, pH 7.6, 1 % DMSO, and 10 pmol of β -Gal. Assays were initiated by addition of resorufin- β -galactopyranoside to a final concentration of 50 μ M. Formation of resorufin was measured using fluorescence, with excitation at 571 nm and recording emission at 584 nm. For enzyme tethered to beads, 18 - 20 mg of beads were suspended in 1 mL of buffer in a 1.5 mL Eppendorf tube with shaking. After 1 min of shaking, the beads were allowed to settle before a 750 μ L aliquot was transferred to a cuvette and the fluorescence measured. The aliquot was then transferred back to the Eppendorf tube and shaken for a further 1 min and the process repeated. Typically 10 time points were recorded for each rate measurement.

4.2.5 Measuring the Concentration of Surface Tethered Enzymes

For determining enzyme concentrations in free solution, the absorbance at 280 nm was measured and concentrations calculated assuming $\epsilon = 110130 \text{ M}^{-1}\text{cm}^{-1}$ based on the amino acid composition of β -Gal. For enzymes immobilized on glass beads the amount of protein bound was determined using sodium bicinchoninate. Reagent A was prepared by dissolving 8 g sodium carbonate monohydrate, 1.6 g sodium tartrate in water to a final volume of 100 mL, pH 11.25.

Reagent B was prepared by dissolving 4 g of sodium bicinchoninate in water to a final volume of 100 mL. Reagent C was prepared by dissolving 0.4 g of copper sulfate heptahydrate in water to a final volume of 10 mL. Reagents A, B, and C were mixed at a ratio of 25:25:1 (v/v/v) just before use. 50-100 mg of beads was mixed with 500 μ L MilliQ water and 500 μ L of the reagent mixture. After vortexing, the mixture was incubated at 60 °C for 15 min. Following incubation, the mixture was allowed to cool to room temperature, before reading the UV absorbance at 562 nm.

4.2.6 Thermal Stability Measurements

To examine the thermal stability of β -Gal variants in free solution, 100 μ L aliquots of a solution containing 100 nM β -gal in 100 mM potassium phosphate buffer, pH 7.6 were heated at temperatures ranging from 25 - 60 °C for 10 minutes using a thermocycler, followed by a rapid cooling to room temperature. The enzyme solution was diluted to 10 nM prior to the assaying for residue enzyme activity at 25 - 60 °C.

To examine the thermal stability of β -gal variants tethered to glass beads, 18 – 20 mg aliquots of enzyme-functionalized beads, corresponding to 10 pmol of enzyme, were suspended in 100 μ L of 100 mM potassium phosphate pH 7.6 and were heated and cooled as described. The bead suspension was diluted with 900 μ L of room temperature buffer was added prior to assay for residual enzyme activity. To estimate T_m , and the slope of the curve at T_m , thermal stability data were fit to equation 1 as described previously³

$$y = A_2 + \frac{A_1 - A_2}{1 + \exp\left(\frac{T_m - T}{\text{slope}}\right)}$$

Where A_1 and A_2 are the upper and lower asymptotes, respectively, of the enzyme activity and T_m is the temperature at which 50% of the initial activity remains.

4.3 Results

4.3.1 Varying Maleimide Surface Density

Alkyne functionalized surfaces were incubated overnight with solutions containing varying molar ratios of azide-PEG-maleimide chains to azide-PEG chains terminated either with a hydroxyl group or with a methyl group. Through ‘click chemistry’, this incubation generated glass surfaces derivatized with varying amounts of maleimide.

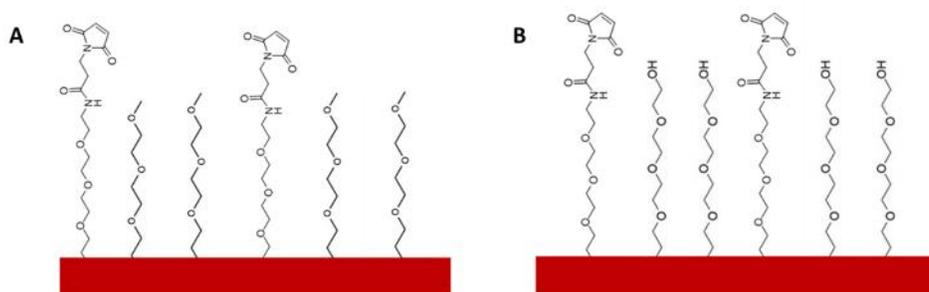


Figure 4.1: Spacing maleimide terminated linkers with a) methyl terminated linkers or b) hydroxyl terminated linkers

Maleimide-derivatized surfaces were incubated for four hours with 5 μ M of β -gal, either the V152C construct, or the no-thiol construct. Excess protein was washed with buffer containing 2% Tween to remove non-specific adsorption. Protein concentrations were measured using the MicroBCA assay. These concentration measurements were normalized to the surface area of a 75 μ M glass bead and reported as molecules per 100nm².

In figure 2, protein surface concentration is graphed relative to the molar ratios of maleimide terminated linkers to hydroxyl terminated linkers. For all available surface constructs, the surface concentration of the V152C construct is >2.5 molecules per 100nm², consistent with the formation of a protein monolayer (see Chapter 2). The concentration of V152C remains consistent from a maleimide:OH molar ratio of 1:04 through 1:50. At 1:100, the protein surface concentration decreases, and this decrease continues at the 1:250 surface. This decrease in surface concentration is expected when the available maleimide groups are diluted to a degree that proteins are less able to react with them during a given incubation period.

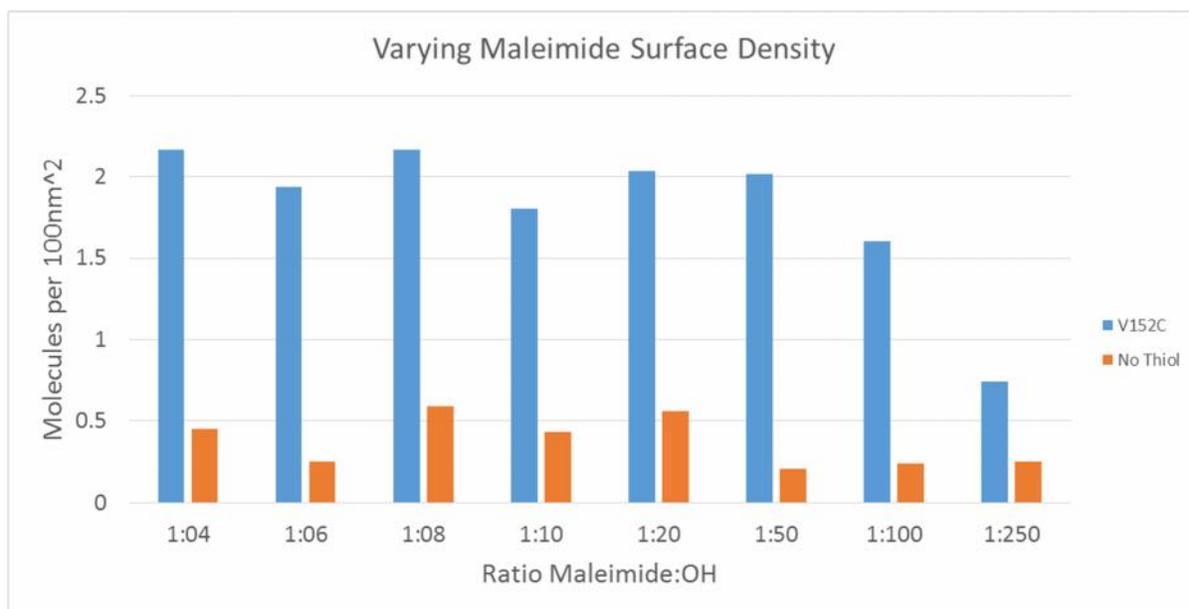


Figure 4.2: Protein Surface Density vs. Maleimide/Hydroxyl Molar Ratio

In figure 3, protein surface concentration is again plotted, this time relative to the molar ratios of maleimide terminated linkers to methyl terminated linkers. For all available surfaces,

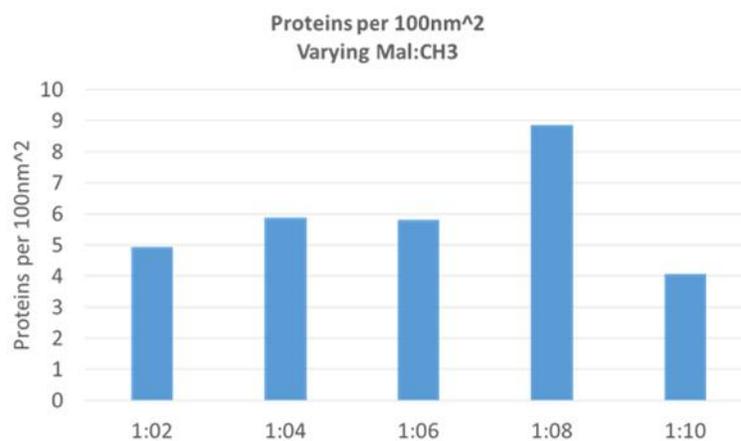


Figure 4.3: Protein Surface Density vs. Maleimide/Methyl Molar Ratio

the surface concentration of V152C greater than 4 molecules per 100nm², and in most cases greater than 6 molecules per 100nm². These values far exceed the concentrations expected for a monolayer, and suggest that there is non-specific adsorption to the hydrophobic surface, and likely the proteins on the surface are unfolded.

The specific activity of the immobilized V152C construct was measured for the maleimide:hydroxyl terminated and maleimide:methyl terminated surfaces. The activity of the surface-bound enzyme was normalized to the activity of the enzyme free in solution. As shown in figure 4, the specific activity increases for the tethered enzyme as the surface maleimide density is diluted, both for the hydrophilic and for the hydrophobic surface. For a given density of maleimide, however, the amount of activity remaining for the immobilized enzyme was higher when tethered to a more hydrophilic surface.

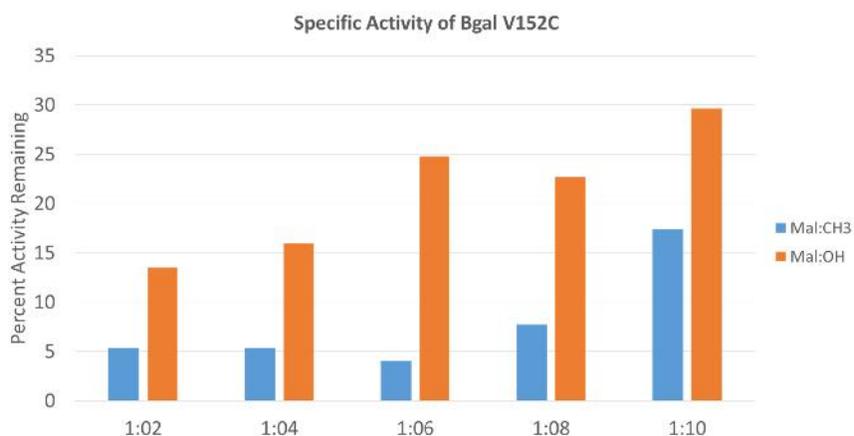


Figure 4.4: Specific Activity For β -gal V152C on Mal:OH and Mal:CH3 Surfaces

4.3.2 Varying Hydrophobicity

In the previous section, it was shown that decreasing the surface maleimide concentration increased the specific activity of the immobilized α -gal, even for hydrophobic surfaces. To probe the effects of hydrophobicity further, surfaces were prepared such that the molar ratio of maleimide terminated linker to non-maleimide terminated linker were kept constant at 1:10. The ratio of hydroxyl to methyl terminated linkers varied to keep the total concentration of non-maleimide linker constant while varying the relative amounts of hydroxyl groups to methyl groups.

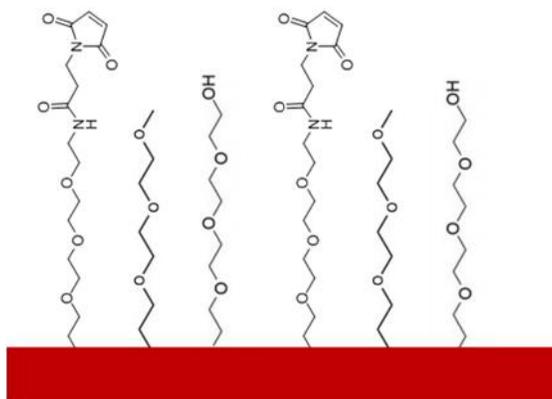


Figure 4.5: Varying hydrophobicity with a constant maleimide:non maleimide molar ratio

In figure 6, the percent of specific activity of α -gal remaining after tethering to the surface is plotted relative to the surface hydrophobicity. The activity of both the V152C and the no-thiol constructs were measured. For the covalently tethered V152C construct, the specific activity decreases as the hydrophobicity of the surface increases. For the adsorbed no thiol construct, the specific activity is consistently low, suggesting that adsorbed protein is likely denatured. There is a slight increase in specific activity with increasing hydrophobicity of the

surface. This is likely due to increased adsorption of protein on surfaces that are more hydrophobic.

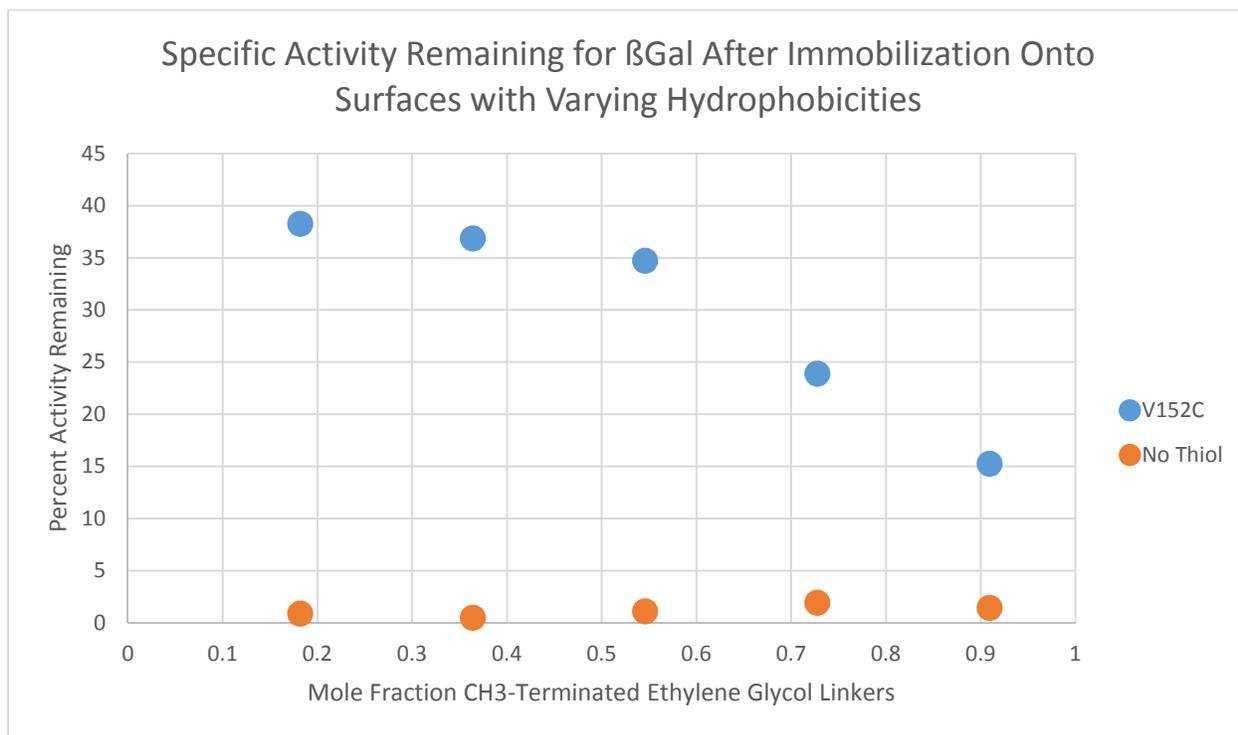


Figure 4.6: Percent Activity Remaining for β -gal Tethered to Surfaces with Increasing The ratio of molar ratio of maleimide to non-maleimide remained constant at 1:10. The mole fraction of CH₃ – to OH- terminated ethylene glycol linkers was varied

In Chapter 3, it was shown that immobilizing β -gal to a fully maleimide-terminated surface can lead to a decrease in thermal stability. This decrease likely occurred because of unfavorable protein-surface interactions. Since the maleimide group is slightly hydrophobic, this would suggest that by increasing the hydrophilicity of the surface, the thermal stability of the V152C construct should increase the thermal stability. To test this hypothesis, we compared the thermal stability curves of the V152C construct and the E147C construct on fully maleimide-

terminated surfaces with the thermal curve of these constructs on a hydrophilic surface – one with a 1:10 maleimide:hydroxyl terminated makeup.

Beads functionalized with α -gal were heated for 10 minutes to a temperature between 24°C and 65°C using a thermocycler. These beads were then cooled to 24°C and resuspended in room temperature buffer. The activity for each temperature was measured and normalized to the activity for beads heated to 24°C.

As seen in figure 7, the hydrophilicity of the surface had little effect on the immobilized E147C construct. Since the E147C construct tethers α -gal to the surface via a rigid α -helix, the protein surface contact would be minimized. This result agrees with our findings from Chapter 3, where rigid attachment sites minimize protein surface interactions.

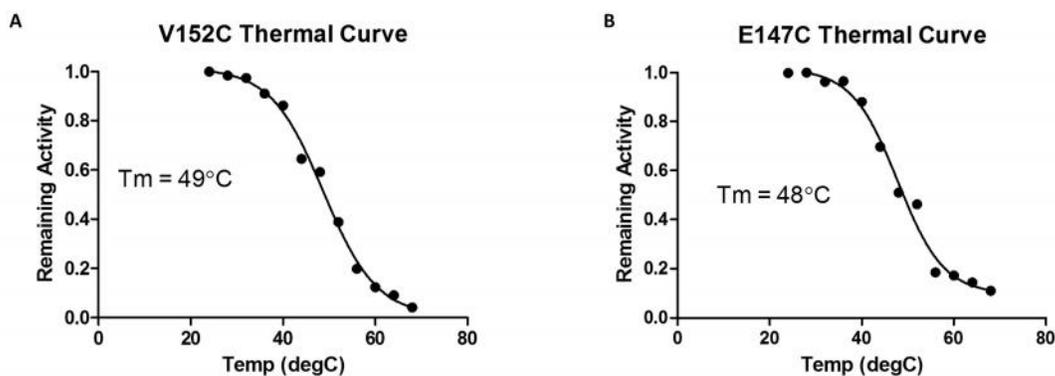


Figure 4.7: Thermal Melt Curves for V152C and E147C on a 1:10 Maleimide:OH surface

For the V152C construct, the thermal stability increased from 38°C on a full maleimide surface to 49°C on the hydrophilic surface. The thermal stability of V152C construct on the hydrophilic surface is virtually identical to the thermal stability of the immobilized E147C

construct and only a few degrees less than the thermal stability of β -gal free in solution. This would suggest that the decreased thermal stability of the V152C construct immobilized to a full maleimide surface was due to hydrophobic interactions between the surface and the protein, which is consistent with our proposed hypothesis from Chapter 3.

4.3.3 Varying Surface Charge

In addition to hydrophobicity, we wanted to examine what role surface charge may have on the activity and thermal stability of surface-tethered enzymes. Surfaces were once again prepared where the molar ratio of maleimide terminated linkers to non-maleimide terminated linkers were kept constant. The non-maleimide terminated linkers consisted of varying ratios of amine terminated to carboxy terminated linkers.

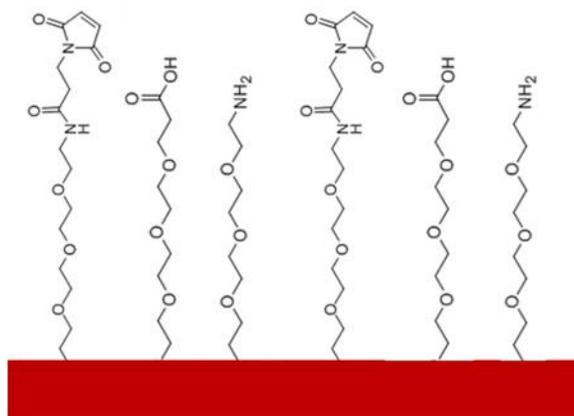


Figure 4.8: Varying the surface charge by varying the NH₃:COOH molar ratio, but keeping the maleimide:non maleimide molar ratio constant at 1:10

In figure 9, the protein surface concentration, reported as molecules per 100nm², is plotted for surfaces of varying charge. For the V152C construct, the protein surface

concentration is consistently less than 1 molecule per 100nm², which is consistent with a protein sub-monolayer coverage – see Chapter 2. As the surface becomes increasingly negative, the concentration tends to decrease. This is expected, given that β -gal has a net negative charge at pH 7.5. Interestingly, the protein surface concentration is less for the charged surfaces than for the hydrophilic surfaces, as reported in figure 9. For the no-thiol construct, the surface concentration is always less than 0.1 molecules per 100nm², much less than 10% of the measured concentration of V152C.

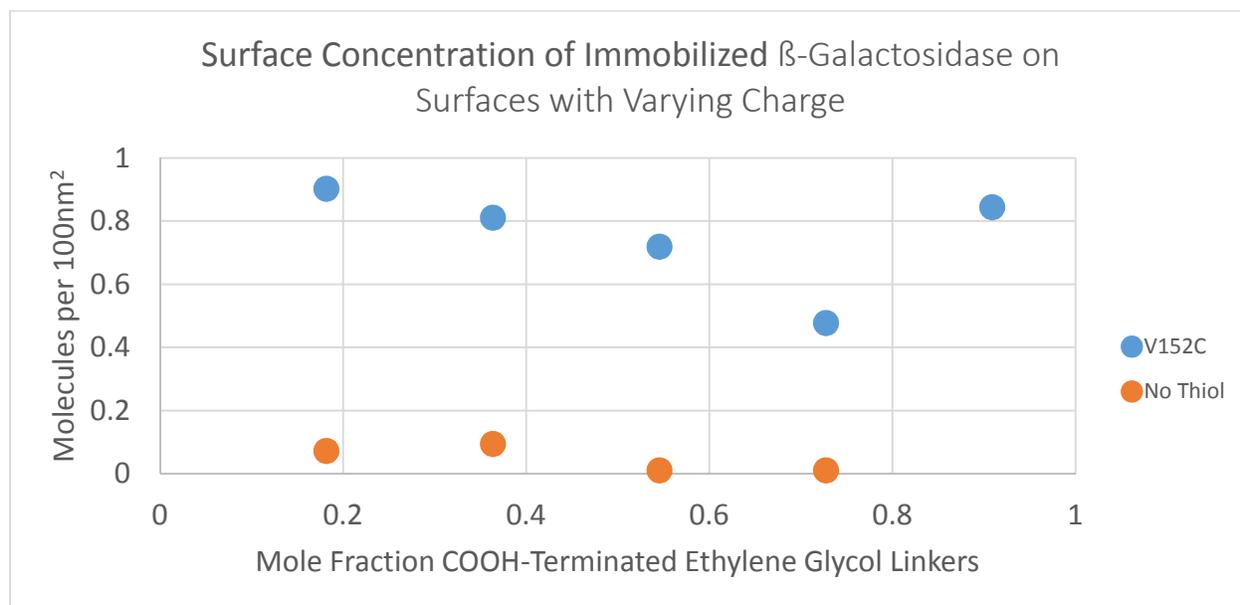


Figure 4.9: Protein surface concentration for surfaces with varying charge. The ratio of molar ratio of maleimide to non-maleimide remained constant at 1:10. The mole fraction of NH₃- to COOH- terminated ethylene glycol linkers was varied

The specific activity of the V152C construct was plotted for surfaces with different charges. As shown in figure 10, β -gal immobilized onto surfaces with mixed charges retained

more activity than that immobilized onto surfaces that were either more negatively charged or more positively charged surface. The decrease in retained activity was less as the surface became more negative, while more of the activity was lost for positive surfaces.

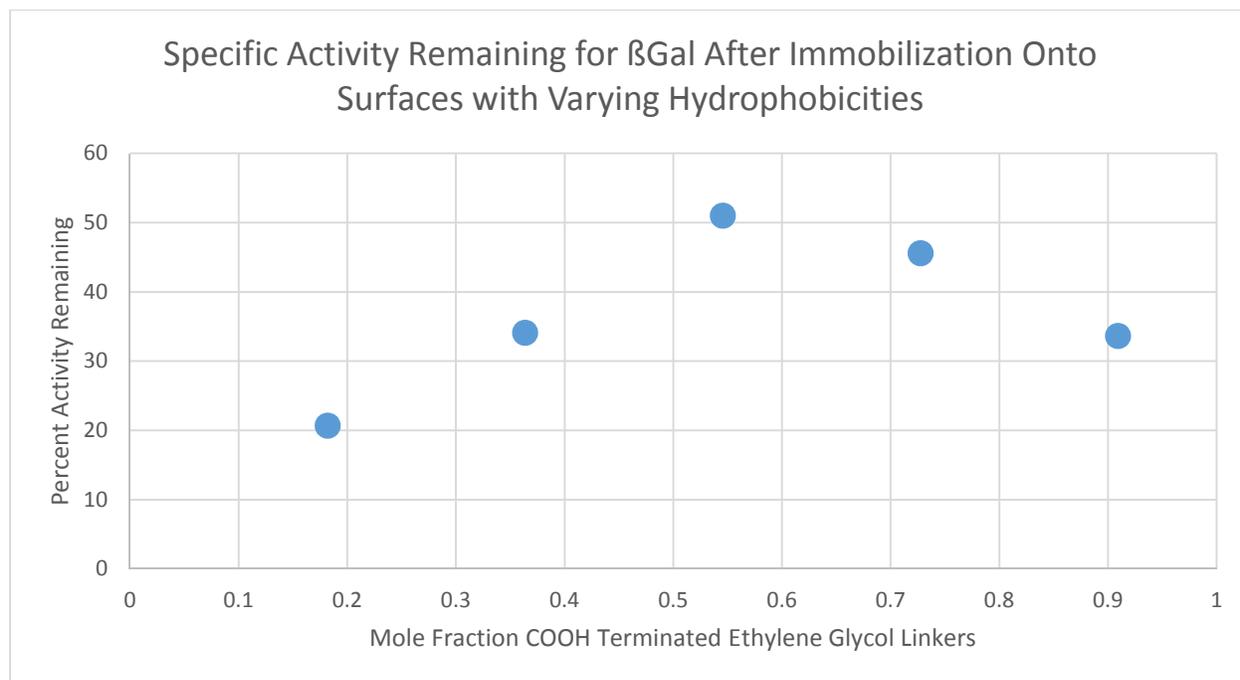


Figure 4.10: Activity of β -gal V152C immobilized on surfaces with varying charge. The ratio of molar ratio of maleimide to non-maleimide remained constant at 1:10. The mole fraction of NH_3^- to COOH^- terminated ethylene glycol linkers was varied

Next we measured the thermal stability of β -gal on three different surfaces – a negatively charged surface, a positively charged surface, and a surface with mixed charges. For all three surfaces, the molar ratio of maleimide to non-maleimide was 1:10. The V152C construct was immobilized onto each of these surfaces, and the thermal stability measurements proceeded as described previously in this chapter.

Figure 11 shows the thermal stability curves for these three surfaces. Consistent with the activity assays described in figure X, the mixed charge surface was the most stable. On this mixed charge surface, the T_m 46°C, only 4°C below the measured T_m of -gal free in solution. The positively and negatively charged surfaces were both destabilizing, though the T_m of V152C tethered to the negatively charged surface was 3°C higher than V152C tethered to the positive surface.

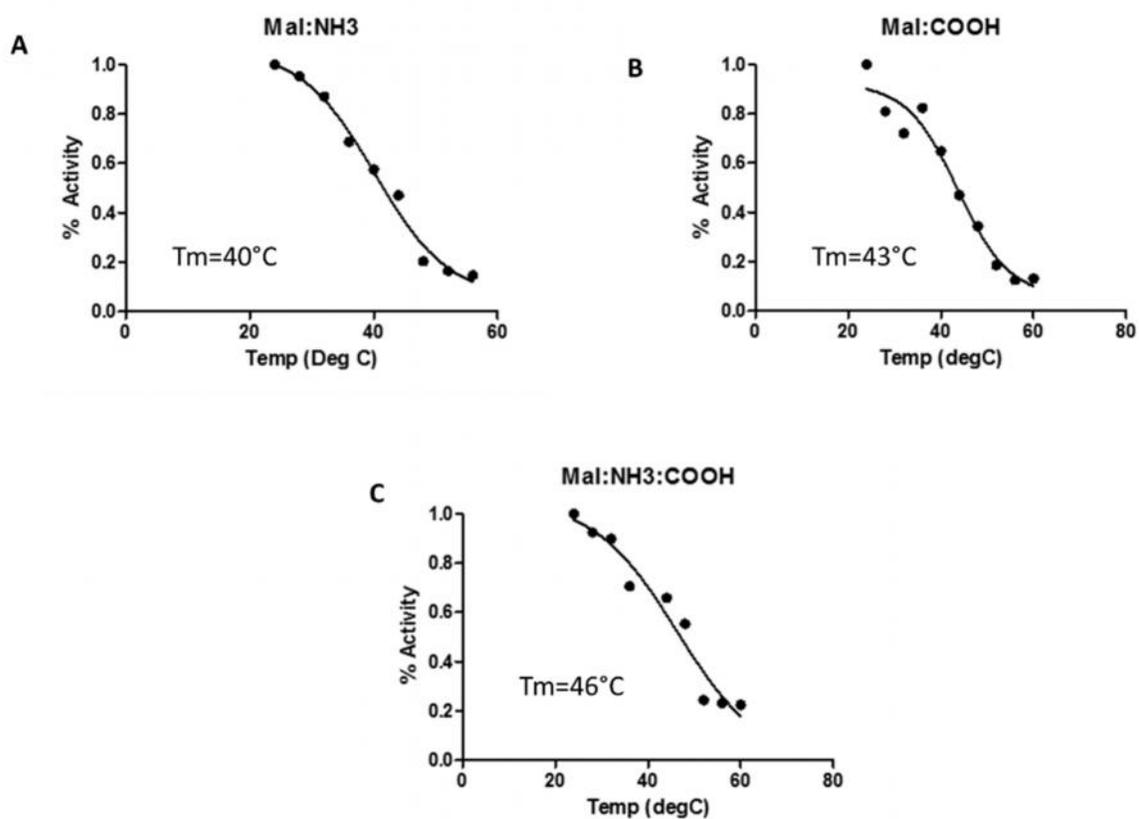


Figure 4.11: Thermal stability curves for 1:10 mal:NH3, 1:10 mal:COOH, and 1:6:4 mal:COOH:NH3

4.4 Discussion

Much work has been done concerning the non-specific interactions that proteins have with surfaces. Researchers have examined the role that hydrophobicity⁴⁻⁶ and electrostatic charge^{7,8} has on non-specific protein adsorption to surfaces. More recently, it has been shown that attaching enzymes to surfaces via a unique, chemically defined covalent linkage results in the immobilized enzyme retaining more of the specific activity relative to free solution⁹.

An unexplored question that the work in this chapter examined is how non-specific interactions affect enzymes that are tethered to surfaces via unique, chemically defined linkages. Presumably, given the proximity of the covalently immobilized enzyme to the surface, the possibility exists for non-specific interactions between the protein and the surface. We hypothesize that these interactions explain why enzymes covalently attached to surfaces via a unique linkage still exhibit reductions in specific activity relative to free solution. In Chapter 3, for example, it was hypothesized that these non-specific protein-surface interactions play a crucial role in the thermal stability of immobilized enzymes. We explored this idea further by altering the hydrophilic and electrostatic properties of the surface, and measuring the resulting activity and stability.

When the hydrophilicity of the surface increases, the specific activity and thermal stability of the immobilized enzyme increases. This is consistent with the results of Chapter 3. The data suggests that hydrophobic protein surface interactions are disruptive, and increasing the hydrophilicity of the surface can disrupt these interactions.

A mixed charge surface also increases the activity of the immobilized enzyme. Taken with the results from the hydrophilic surfaces, this suggests that protein surface interactions are

inherently disruptive to protein stability and to specific activity. Indeed, although tethering the enzyme to a negatively charged surface resulted in a slight reduction in specific activity relative to the mixed charge surfaces, this reduction was small compared to the reduction when β -gal was immobilized onto the positively charged surface. Because β -gal is negatively charged under the experimental conditions, one can imagine that significant electrostatic interactions between the protein and the positively charged surface may disrupt the native structure.

Interestingly, enzyme activity was higher on a mixed charge surface as compared to a hydrophilic uncharged surface. As discussed in Chapter 1, it was found by a number of researchers that hydroxyl terminated surfaces can still promote some degree of non-specific adsorption, while zwitterionic surfaces were shown to be more resistant to adsorption. These observations are consistent with our results – the “no thiol” β -gal construct adsorbed to a lesser degree on the charged surfaces. Given that these proteins were tethered to flexible loops, the protein is free to adopt a wide variety of orientations relative to the surface. For the uncharged hydrophilic surface, the enzyme is more likely to maintain some of the more unfavorable orientations relative to the surface. For a charged surface, however, the electrostatic repulsion may drive the protein to retain a more rigid conformation, so as to minimize protein surface contact.

The thermal stability of immobilized β -gal never increased beyond the stability of β -gal free in solution. This was an interesting result, since the literature often cites increased stability as a reason for enzyme immobilization. It is often hypothesized that this stabilization is due to the surface blocking a portion of the enzyme unfolding pathway, thereby decreasing the likelihood of unfolding. A flat surface, however, only blocks a small volume of space surrounding the immobilized enzyme. Any entropic gain from this, therefore, is likely to be

small. Indeed, it is interesting to note that many papers report increased stability when the protein is either immobilized into a porous material or trapped within a polymer matrix. This would suggest that immobilization itself is not stabilizing, but rather that crowding provides stability, and that a significant volume surrounding the protein must be crowded before any tangible increases in stability are observed.

4.5 References

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Chapter 5

The Influence of Orientation on the Activity of Surface Tethered Enzymes

5.1 Introduction

The work in this chapter is the result of a collaboration. The SFG spectra was gathered by Yaoxin Li, and the molecular dynamics simulation was performed by Shuai Wei.

When non-specifically tethering enzymes to surfaces, whether through adsorption or through covalent crosslinking to surface lysine residues, both orientation and unfolding are cited as reasons why specific activity decreases. Non-specific attachment of the protein to the surface generally results in multiple non-specific interactions of the surface with the same protein molecule that may not be favorable to the structural integrity of the enzyme^{1,2}. In addition, a portion of the population of immobilized enzymes will have the active site oriented towards the surface, rendering the active site inaccessible to substrate in the bulk solution.

Because non-specific attachment may lead to both an unfolding of the protein and an unfavorable orientation simultaneously, very little work has been done to examine the two hypotheses separately. A number of studies have shown that having a single attachment site allows the immobilized enzyme to retain a larger proportion of activity vs. non-specific attachment², however, this single attachment site is frequently located either at the N- or C-

terminus³. Little work has been done on deliberately orienting the active site relative to the surface.

In chapters 3 and 4, we examined how the chemical nature of the surface can influence the structural integrity of β -galactosidase. In this chapter, the role of the orientation of the active site on the specific activity of the enzyme was examined. Three constructs of β -Gal have been tested with two different orientations of the active site. The V152C construct places the cysteine on a loop region distal to the active site, thus orienting the active site towards the bulk solution. The E227C and D308C constructs place the cysteine on loop regions near the active site, thus orienting the active site towards the surface.

We hypothesized that orienting the active site of the immobilized enzyme towards the surface will reduce the specific activity to a greater degree than if the active site was oriented towards the bulk solution. Whereas the data appear to agree with this hypothesis, we found that the degree to which the hypothesized orientation affected activity was in fact more heavily influenced by distribution of orientations with respect to the surface allowed by the choice of attachment point.

5.2 Materials and Methods

5.2.1 Materials

75 μ m acid washed glass beads were purchased from Sigma Aldrich. Alkyne triethylsilane was purchased from Gelest. Azido-PEG₃-Maleimide was purchased from Click Chemistry Tools. Azido-PEG₃-OH and Azido-PEG₃-COOH were purchased from Conju-Probe. Resorufin- β -galactopyranoside was obtained from Life Technologies (Grand Island, NY).

5.2.2 Functionalizing Glass Beads with Self Assembled Monolayers

Acid washed glass beads were shaken overnight at room temperature with 1mM of alkyne triethylsilane in anhydrous toluene. After washing three times with toluene to remove excess alkyne triethylsilane, the surface was shaken overnight at room temperature with 10mM of azido- Azido-EG₃ – X, where X could be maleimide, hydroxyl, amide, or carboxylic acid. Excess Azido-EG₃ – X was washed 3x to remove excess linker. The beads were then dried using a vacuum and stored in a desiccator at 4°C until use.

5.2.3 Functionalizing Protein to Maleimide

Stock solutions of β -gal were incubated with 1mM of TCEP for 20 minutes to reduce any disulfide bonds that may have formed. Maleimide derivatized beads were incubated with 5 μ M of reduced β -gal for 4 hours at room temperature with gentle shaking. Excess protein was washed 3x with buffer + 2% Tween20 following a wash 3x with buffer. Protein-functionalized beads were used immediately.

5.2.4 Assaying the Activity of Surface Tethered β -Gal

Enzyme activity was determined using the fluorogenic substrate resorufin- β -galactopyranoside (Life Technologies, Grand Island, NY). All assays were performed in 1 mL of buffer containing 100 mM potassium phosphate, pH 7.6, 1 % DMSO, and 10 pmol of β -Gal. Assays were initiated by addition of resorufin- β -galactopyranoside to a final concentration of 50

μM . Formation of resorufin was measured using fluorescence, with excitation at 571 nm and recording emission at 584 nm. For enzyme tethered to beads, 18 - 20 mg of beads were suspended in 1 mL of buffer in a 1.5 mL Eppendorf tube with shaking. After 1 min of shaking, the beads were allowed to settle before a 750 μL aliquot was transferred to a cuvette and the fluorescence measured. The aliquot was then transferred back to the Eppendorf tube and shaken for a further 1 min and the process repeated. Typically 10 time points were recorded for each rate measurement.

5.2.5 Measuring the Concentration of Surface Tethered Enzymes

For determining enzyme concentrations in free solution, the absorbance at 280 nm was measured and concentrations calculated assuming $\epsilon = 110130 \text{ M}^{-1}\text{cm}^{-1}$ based on the amino acid composition of β -Gal. For enzymes immobilized on glass beads the amount of protein bound was determined using sodium bicinchoninate. Reagent A was prepared by dissolving 8 g sodium carbonate monohydrate, 1.6 g sodium tartrate in water to a final volume of 100 mL, pH 11.25. Reagent B was prepared by dissolving 4 g of sodium bicinchoninate in water to a final volume of 100 mL. Reagent C was prepared by dissolving 0.4 g of copper sulfate heptahydrate in water to a final volume of 10 mL. Reagents A, B, and C were mixed at a ratio of 25:25:1 (v/v/v) just before use. 50-100 mg of beads was mixed with 500 μL Millipore water and 500 μL of the reagent mixture. After vortexing, the mixture was incubated at 60 $^{\circ}\text{C}$ for 15 min. Following incubation, the mixture was allowed to cool to room temperature, before reading the UV absorbance at 562 nm.

5.3 Results

5.3.1 The Effect of Orientation on Hydrophilic Uncharged Surfaces

Hydrophilic surfaces were prepared by incubating alkyne derivatized glass beads with a 1:10 molar ratio of maleimide terminated ethylene glycol linkers to hydroxyl terminated PEG linkers. Three of the available β -Gal constructs – V152C, E223C and D308C – were used in these experiments, as shown in figure 1.

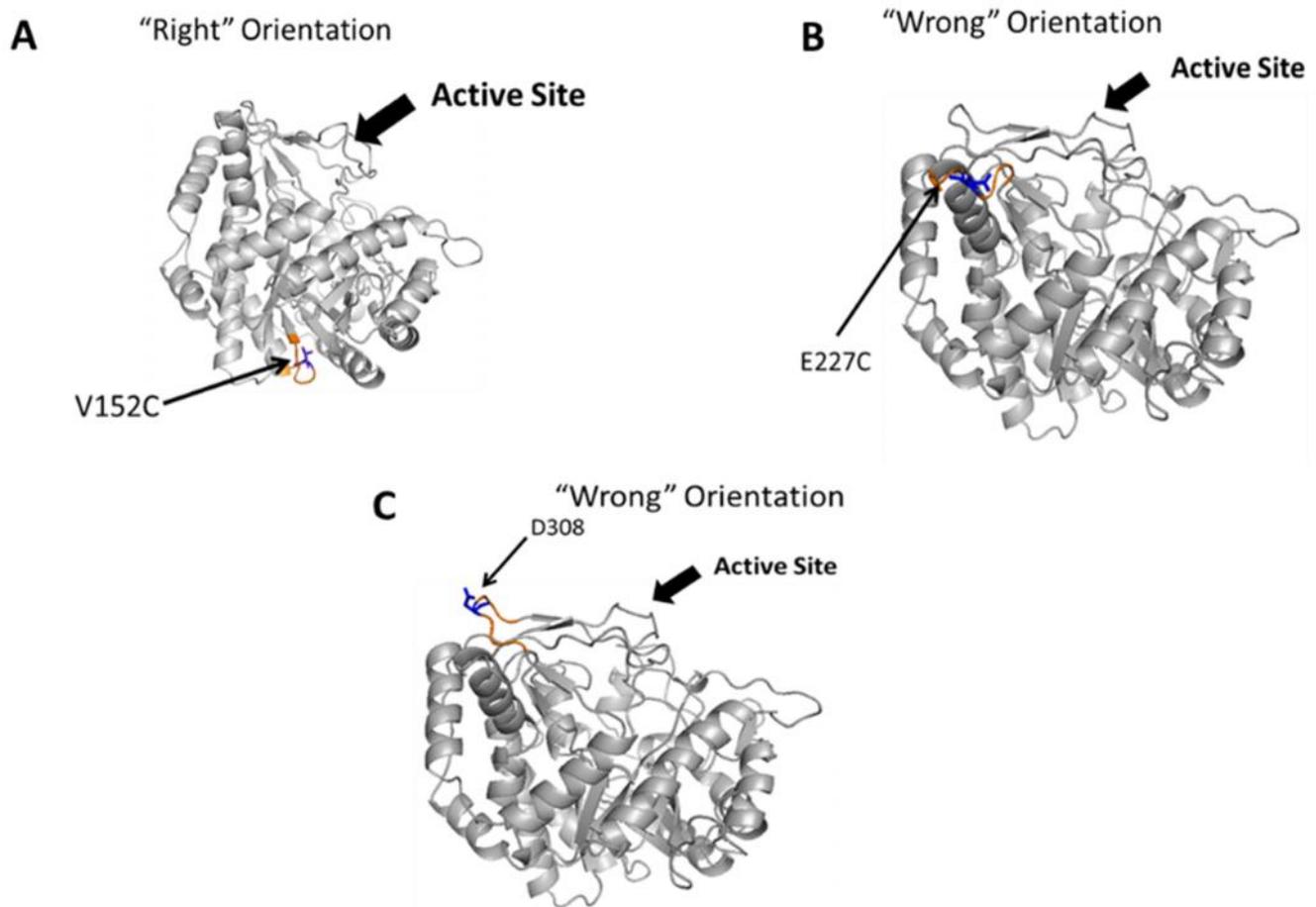


Figure 5.1: The three β -Gal constructs used in this chapter

Once any excess protein was washed away, the surface concentration was measured using the microBCA assay. These measurements were normalized to the available surface area of the beads and reported at molecules per 100nm^2 . As seen in figure 2, the surface concentration for all three constructs were well within the definition of a surface monolayer, as defined in chapter 2.

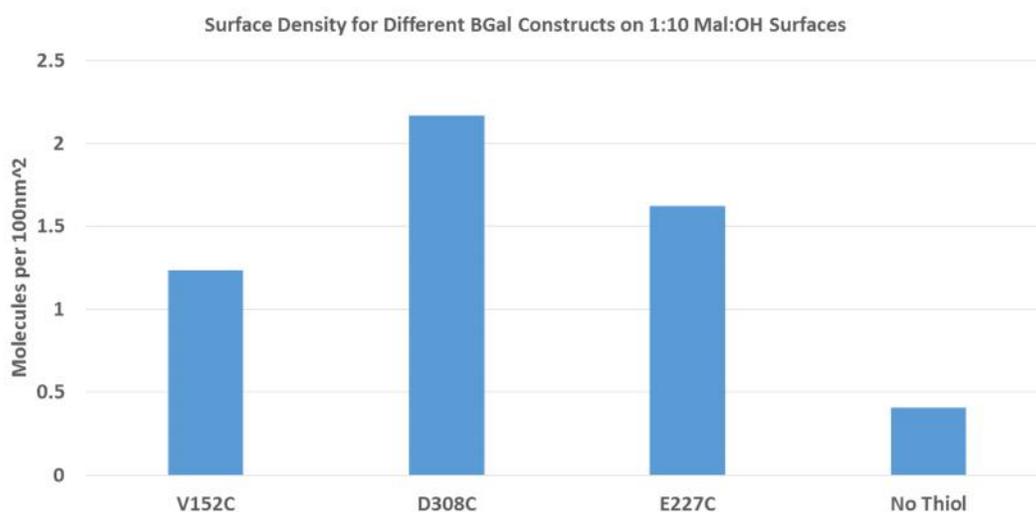


Figure 5.2: The surface concentration density for the three β -gal constructs tethered to a mixed maleimide-hydroxyl terminated surface. The no thiol construct is a control

The specific activity of the immobilized enzyme was measured using the fluorescent substrate resorufin- β -galactopyranoside (RBG). β -galactosidase will hydrolyze the ether bond of RBG to release galactose and the fluorescent molecule resorufin. The production of resorufin was measured using an excitation wavelength of 571nm and an emission wavelength of 584nm.

The measured specific activity of the immobilized enzyme was normalized to the activity of the same construct in solution, and reported as 'Percent Activity Remaining'.

The results are shown in the orange bars in figure 3. The V152C construct, when tethered to the surface, retains a larger proportion of the specific activity relative to free solution than either the E227C or the D308C construct. The immobilized V152C retains $26 \pm 2.9\%$ of specific activity relative to free solution, while E227C retained $17 \pm 2.2\%$ and D308C retained $16 \pm 1.5\%$. The V152C construct, with the active site oriented towards the bulk has a statistically higher specific activity than either E227C or D308C.

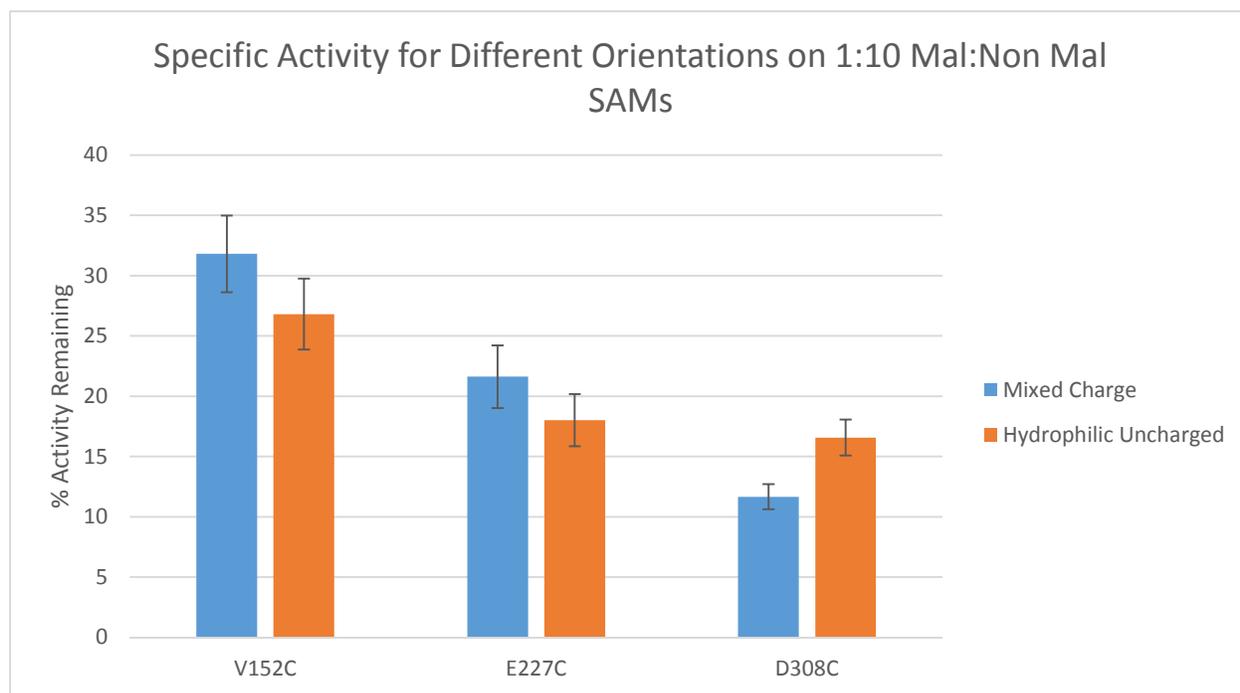


Figure 5.3: The specific activity of β -gal retained after immobilization to either noncharged hydrophilic surfaces (blue bars) or charged surfaces (orange bars)

In chapter 4, it was shown that charged surfaces can reduce non-specific adsorption of β -gal as compared to a hydrophilic uncharged surface. For a protein tethered to a flexible linker, the propensity for non-specific adsorption may lead to the stabilization of orientations other than predicted through the placement of the mutation. A certain proportion of the population may lay on its side, for example, rather than have the active site oriented either towards the bulk or towards the surface.

To examine this hypothesis, we tethered the same three constructs to a 1:6:4 maleimide:COOH:NH₃ surface, prepared as described in chapter 4. This surface was chosen because this charge combination led to the highest retention of specific activity. The specific activity of the three immobilized constructs are shown in the blue bars in figure 3. The retained specific activity for immobilized V152C and E227C relative to free in solution remained within error for both surfaces. For V152C, the retained specific activity was $31 \pm 3.2\%$ for the mixed charge surface, while E227C had a retained specific activity of $21 \pm 2.6\%$. For the D308C construct immobilized onto the charged surface, however, the difference in retained activity relative to the uncharged hydrophilic surface was significant. For the mixed charge surface, the retained specific activity was measured to be $11 \pm 1.3\%$

5.3.2 SFG Data of Different Orientations

Yaoxin Li, a graduate student from Zhan Chen's Lab, performed the SFG experiments for the three β -Gal constructs in this chapter, V152C, E227C, and D308C. Right angle prisms functionalized with an alkyne-silane monolayer were incubated with a 1:10 molar ratio of maleimide-PEG₄-azide to hydroxyl-PEG₄-azide, and click chemistry was used to attach the monolayer.

The protein constructs were tethered to the surface using the same protocol as outlined for the activity assays. The SFG spectra of two polarized IR beams were collected, and the ratio of the two spectra was used to estimate the net orientation, as outlined previously⁴.

The SFG spectra for the three constructs tethered to the hydrophilic surface are shown in figure 4. As seen in figure 4a, the spectra of the V152C construct has a ratio of 1.93. This is in close agreement with the previously published value of 1.95⁴. This suggests that the orientation of the V152C construct tethered to the hydrophilic surface likely matches the published orientation, which is consistent with the hypothesized orientation – i.e. that the active site is oriented towards the bulk.

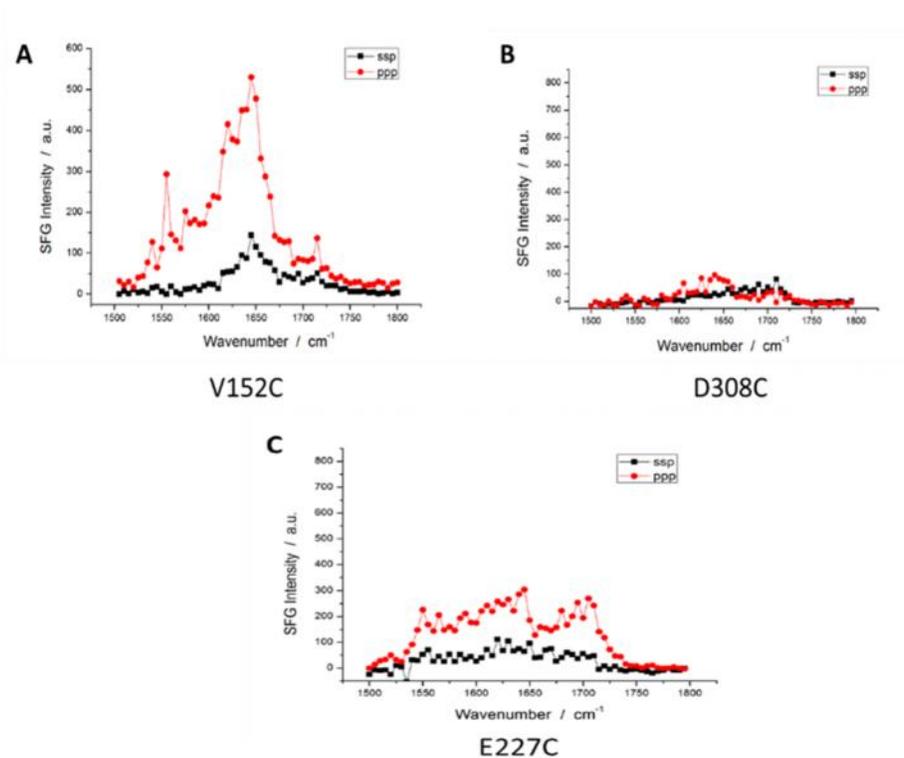


Figure 5.4: SFG Spectra for the three -Gal constructs immobilized onto a hydrophilic surface

In figure 4b,c the SFG spectra of the E227C and D308C constructs tethered to the hydrophilic surface are shown. In both cases, no measurable SFG spectra was detected. One

possible explanation might be that some protein has randomly adsorbed onto the surface, thereby reducing the strength of the signal. If this were true, however, it would also have occurred with the V152C construct. Also, as discussed in Chapter 4, the no thiol construct had a very low surface concentration, suggesting that non-specific adsorption is not significant. Another possibility might be that the D308 and E227C constructs unfold upon adsorption. The surface electrostatic charge of β -gal, as generated by Pymol using the PDB# 2PBG crystal structure is shown in Chapter 2. This figure shows that the surface charge is fairly uniform, and uniformly negative. There are no uniquely charged residues near either the D308C or E227C tethering site to suggest that the surface interactions would vary significantly from the V152C attachment site.

This leaves two possibilities: either the orientation of the enzyme lies in a region where SFG is not sensitive enough to detect, or the enzymes are occupying multiple orientations on the surface, and none are predominant enough to generate a strong signal. In either case, this suggests that for the E227C and D308C constructs, some of the enzymes on the surface likely occupy an orientation such that the active site is perpendicular to the surface, rather than facing the surface, meaning that a portion of the immobilized D308C and E227C constructs would have active sites that are available to the bulk solution. This would mean that, though the overall activity of the two constructs would be lower than that measured for V152C, there would still be measurable activity. This is consistent with our measured specific activity.

5.3.3 Simulation of β -Gal E227C and D308C Tethered To Surfaces

Molecular Dynamic simulations of α -Gal E227C and D308C tethered to a surface was performed by Shuai Wei. The methods used for these simulations are discussed in more detail in Chapter 3.

Figure 5 shows a simulation of α -Gal D308C tethered to a flat surface. The orientation of the protein has rearranged such that the active site is exposed to the bulk solution. This is consistent with the hypothesis raised in the previous two sections: that the E227C and D308C constructs are capable of occupying multiple orientations on the surface, with some of these orientations exposing the active site to the bulk solution.

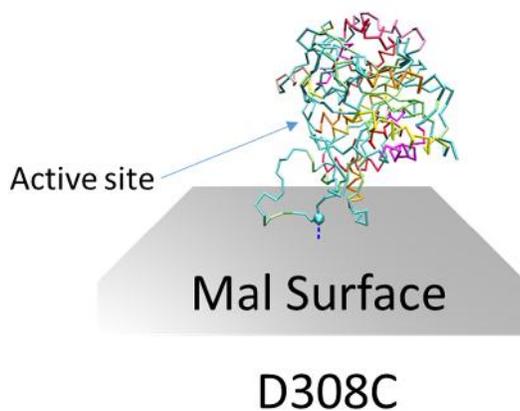


Figure 5.5: A molecular dynamics simulation of D308C tethered to a surface

5.4 Discussion

We hypothesized that orientation played a role in the loss of activity for surface tethered enzymes, specifically, that enzymes with the active site oriented towards the surface will lose more activity than enzymes with the active site oriented towards the bulk solution.

As seen in figure 3, orientation does indeed play a measurable role in the specific activity of surface tethered enzymes. The β -Gal V152C construct, with the active site oriented towards the bulk solution, consistently retained a higher specific activity than the E227C or D308C constructs. This was true for both the hydrophilic uncharged surface as well as the mixed charge surface.

Interestingly, however, the E227C and D308C constructs still retained a measurable level of specific activity. The experimental data demonstrate that orientation alone is not enough to prevent substrate entry into the active site. One possible explanation for this is suggested by the simulation results presented in section 5.3.3: tethering enzymes to flexible loop regions allow the enzyme to occupy a broad range of orientations, some of which will expose the active site to the bulk solution. However, it may also simply be that substrate diffusion into and out of the active site is not sufficiently altered for immobilized β -gal with active sites oriented towards the surface.

The differences in the amount of specific activity retained for different surfaces are also interesting. As shown in Chapter 4, the non-specific adsorption of β -Gal is less favorable on charged surfaces than on hydrophilic but uncharged surfaces. This would suggest that any orientations that increase the likely contact between the protein and the surface is minimized for charged surfaces. For the constructs used in this research: V152C, D308C, and E227C, the minimal protein surface contact is achieved when the enzyme orientation is closest to the hypothesized orientation.

This is reflected in the activity data for the D308C construct, where the level of specific activity retained decreases when tethered to the charge surface. While this agrees with the

hypothesis that the orientation of the active site can play a role, it also suggests that the actual orientation of the enzyme can be influenced by protein-surface interactions.

5.5 References

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Chapter 6

Conclusion and Future Directions

6.1 Conclusion

Understanding how enzymes interact with abiotic surfaces is a key factor in the development of a number of technologies, from catalytic nanoparticles to implantable biomaterials to protein-based biosensors. Of particular interest is the study of the activity and stability of immobilized enzymes. Many researchers have observed that tethering an enzyme to a surface results in reduced specific activity relative to the enzyme free in solution. Less clear are the changes in stability – frequently, researchers report increased stability, though the definition used is not consistent in the literature – often, stability is temporal, where the immobilized enzyme is stored and the specific activity measured over the course of days. The molecular mechanisms underlying these changes in specific activity and stability are poorly understood.

To explore the molecular interactions between the surface and the immobilized enzymes in more detail, β -galactosidase (β -gal) was tethered to a self-assembled monolayer (SAM) constructed on an atomically flat surface. To accomplish this, β -gal constructs were engineered to contain a single surface cysteine. Maleimide-terminated polyethylene glycol self-assembled monolayers were constructed on glass surfaces, and the selectivity of the thiol-maleimide reaction allowed β -gal to be tethered to the surface via a single, chemically-defined linkage.

By placing the tethering site either on a loop or on an α helix, the influence of the attachment site on the thermal stability of the enzyme was examined. By changing the placement of the cysteine relative to the active site, the role of orientation with respect to the

surface on the activity of immobilized enzymes was also studied. Finally, by changing the hydrophobicity or electrostatic charge of the surface, the effects of protein-surface interactions on activity and stability were investigated.

6.1.2 The Effect of Attachment Site on Thermal Stability

Few studies have looked at the correlation between the site where the protein is attached to the surface and the resulting thermal stability of the immobilized enzymes. Most researchers choose an unstructured loop region for immobilization, assuming that tethering a protein to a surface through a flexible loop would be less disruptive to its structural integrity than tethering through a more rigid structural element, such as a helix. Very little data exists to support this assumption. A number of molecular dynamic simulations have previously examined this question (see Chapter 1); however, in most cases, the surface is considered to be 'neutral', meaning that there are minimal interactions between the protein and the surface.

As discussed in the introduction, surfaces are rarely neutral. Electrostatic and van der Waals interactions between proteins and surface can and do occur, and all of these interactions have the potential to influence structural integrity and thereby affect stability. To address this question, we immobilized β -galactoside to a chemically well-defined monolayer through a unique covalent surface attachment.

To determine the thermal stability, β -gal was heated to a particular temperature for 10 minutes, cooled to room temperature, and the remaining activity was measured. The results were quite interesting: for the enzyme tethered to the surface through an attachment on an α -helix, the temperature at which 50% of the activity remained ($T_{1/2}$) was 48°C, within error of the value for

β -gal free in solution. For the construct tethered to the surface via a loop, the $T_{1/2}$ dropped by more than 10°C.

SFG and ATR-FTIR spectra provided by Yuwei Liu in the Chen lab provided some interesting insights. Both the V152C and the E147C constructs, when tethered to the maleimide surface, had the same average orientation. The distribution of orientations, however, was greater for the V152C construct, suggesting that tethering the protein to the surface via a loop increases the available range of motion. This suggests that the increased range of motion can potentially lead to negative protein-surface contacts that would be destabilizing.

To explore this issue further, coarse grain modelling was performed by Shuai Wei of the Brooks lab. These simulations showed that the unfolding of surface-tethered proteins was driven by protein-surface interactions, and that the V152C construct more readily came into contact with the surface, and thus was more unstable.

These results suggest that enzyme immobilization is not always stabilizing, as suggested in the literature. On the contrary, protein-surface interactions clearly play an important role in the relative stability of immobilized enzymes in comparison with enzymes free in solution. For these experiments, the surface consisted entirely of maleimide-terminated PEG linkers. Maleimide is a slightly hydrophobic molecule, which would potentially lead to hydrophobic interactions with the proteins. These hydrophobic interactions would likely lead to the destabilization observed in experiments.

6.1.3 The Effects of Surface Chemistry on Activity and Stability

The results of Chapter 3 suggest that protein-surface interactions can play a role in the stability of immobilized enzymes. To explore this idea further, we tethered β -gal to surfaces where the maleimide terminated PEG linkers were mixed with PEG linkers terminated with chemical moieties to generate surfaces that vary either in hydrophobicity or electrical charge.

By varying the ratio of methyl to hydroxyl terminated PEG molecules (while keeping maleimide surface concentration constant), we were able to generate SAM's with varying hydrophobicity. It was shown that a larger portion of the specific activity of the enzyme was retained on hydrophilic surfaces. The thermal stability of the tethered enzyme was also influenced by surface hydrophobicity. When V152C was tethered to a hydrophilic surface, the $T_{1/2}$ increased from 39°C to 49°C, within error of the $T_{1/2}$ of the enzyme free in solution.

By varying the ratio of amine to carboxylic acid terminated PEG molecules, we were able to generate SAM's with varying charge. The highest level of specific activity retained by the immobilized enzyme was on a surface with a mixture of positive to negative charge. Larger decreases in specific activity were observed for surfaces that were either negatively charged or positively charged, though the degree of loss was larger on a positively charged surface. The same pattern was observed for the values of $T_{1/2}$ as measured on these surfaces.

Taken as a whole, these results suggest that attractive protein-surface interactions are destabilizing, consistent with the observations made on surfaces that consisted entirely of maleimide-terminated PEG SAMs. These results also suggest that repulsive surfaces can

increase the stability of immobilized enzymes by decreasing potential protein surface interactions.

The stability of the enzyme never increased beyond that measured for the enzyme free in solution. This confirms that surface immobilization is not inherently stabilizing. In most cases, immobilized enzymes are destabilized from a variety of attractive protein-surface interactions.

6.1.4 The Influence of Orientation on Activity

In addition to protein-surface interactions, it has been proposed that the orientation of the active site can affect the measured specific activity of immobilized enzymes. To explore this question further, three β -gal constructs were used: the V152C construct used in previous chapters, along with two other constructs, E227C and D308C. In each of those two constructs, the unique solvent accessible cysteine was mutated into a loop near the active site, orienting the active site towards the surface when the enzyme is immobilized.

The specific activity of the three β -gal constructs were measured when tethered to a neutral hydrophilic surface. While the specific activity of V152C was higher than either E227C or D308C, the latter two constructs, when tethered to a surface, retained about 80% of the activity retained by the V152C construct.

To test this further, the actual orientations of these three constructs tethered to these hydrophilic surfaces was determined using SFG. For the V152C construct, the deduced orientation was consistent with the hypothesized orientation. For the D308C and E227C constructs, no SFG signal was detected. As outlined in the discussion section of Chapter 5, we

hypothesize that this lack of signal is caused by enzyme occupying multiple orientations on the surface, many of which expose the active site. This would be consistent with the activity data.

These results suggest that orientation does indeed play a role in the specific activity of immobilized enzyme. However, the actual orientation of the tethered enzyme may vary from the hypothesized orientation. These orientation rearrangements are driven by attractive protein-surface interactions.

6.2 Future Directions

6.2.1 The Effects of Protein Surface Density

One question that has not been addressed in detail is the role that protein surface density plays in the retained specific activity of immobilized enzymes. It has been hypothesized that increasing the number of enzymes on the surface will be destabilizing due to repulsive protein-protein interactions.

As defined in Chapter 2, a β -gal monolayer has a concentration less than 3 molecules per 100nm^2 . However, the specific relationship between surface concentration and retained specific activity has not been explored in detail.

6.2.2 Crowding

It has frequently been suggested in the literature that immobilizing enzymes onto surfaces will lead to increased stabilization. However, the results of these studies show that surface immobilization can often be destabilizing. In the literature, the stabilization of tethered enzymes is attributed to the surface effectively blocking the volume through which the enzyme would unfold.

For the studies in this thesis, the surface is flat, and thus occupies a small portion of the potential unfolding pathway. To explore this phenomenon further, the surface tethered enzyme could be immobilized with other molecules of varying lengths and charges. Short-chain PEG molecules could be used to explore uncharged hydrophilic molecules. In addition, short-chain charged molecules could also be co-immobilized onto the surface with the enzyme. Our previous results suggest that crowding with zwitterionic molecules may also be stabilizing.