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PROBING THE CONFORMATION AND ORIENTATION OF ADSORBED ENZYMES USING SIDE-CHAIN MODIFICATION

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Abstract

The bioactivity of enzymes that are adsorbed on surfaces can be substantially influenced by the orientation of the enzyme on the surface and adsorption-induced changes in the enzyme's structure. Circular dichroism (CD) is a powerful method for observing the secondary structure of proteins; however, it provides little information regarding the tertiary structure of a protein or its adsorbed orientation. In this study, we developed methods using side-chain specific chemical modification of solvent-exposed tryptophan residues to complement CD spectroscopy and bioactivity assays to provide greater detail regarding whether changes in enzyme bioactivity following adsorption are due to adsorbed orientation and/or adsorption-induced changes in the overall structure. These methods were then applied to investigate how adsorption influences the bioactivity of hen egg white lysozyme (HEWL) and glucose oxidase (GOx) on alkanethiol self-assembled monolayers (SAMs) over a range of surface chemistries. The results from these studies indicate that surface chemistry significantly influences the bioactive state of each of these enzymes, but in distinctly different ways. Changes in the bioactive state of HEWL are largely governed by its adsorbed orientation, while the bioactive state of adsorbed GOx is influenced by a combination of both adsorbed orientation and adsorption-induced changes in conformation.

Introduction

Protein-surface interactions play a key role in the success or failure of materials used for a wide variety of applications, ranging from industrial processes to implanted biomaterials.¹⁻⁹ The surface immobilization of biomolecules has become increasingly popular in various fields for several reasons because the process can create interfaces that are molecularly recognized and elicit predictable biological responses. The surface immobilization of enzymes has been used extensively for industrial purposes because immobilization can render them resistant to further conformational changes, allowing for the fabrication of reusable substrates that retain their functionality for longer times and/or in harsher environments than the enzyme in the solution state.¹⁰⁻¹²

The major types of surface immobilization can be characterized as nonspecific noncovalent binding (e.g., electrostatic interactions, hydrophobic interactions, and hydrogen bonding), specific noncovalent binding (e.g. ligand-receptor binding), and covalent binding (e.g., cross-linking and coupling agents).^{2, 12} The noncovalent attachment of biomolecules generally results in the weakest association between the biomolecules and the substrate but

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has the benefit of potential reversibility.^{13, 14} Multipoint covalent immobilization is often used in processes that require elevated temperatures since the rigid fixation of the molecule helps to prevent subsequent conformational changes and enzymatic inactivation.¹⁵ However, it has been reported that excessive covalent bonding can lead to the destabilization of an enzyme's structure, which can compromise its bioactivity.¹⁶

The adsorption behavior of an enzyme is of critical importance for the success of any of the aforementioned immobilization techniques. It is important that the enzymes are adsorbed strongly enough that they remain fixed in place during catalysis, but in a manner that does not inactivate the enzyme. Not only is the surface density of the enzyme an important factor that influences the efficiency of immobilized enzyme systems, but the conformation and orientation of the adsorbed enzymes are both important factors as well. This highlights the need for the development and/or enhancement of techniques to further the understanding of protein-surface interactions at the molecular level.

Circular dichroism (CD) spectroscopy has been used extensively over the past few decades to obtain a quantitative assessment of the secondary structure of biomolecules.^{17–19} CD is an attractive analytical tool due to its speed, sensitivity, rapid data analysis, and capability of analyzing surface adsorbed proteins.^{20–23} However, the usefulness of CD is limited in that it provides very little information about the tertiary structure of proteins and no information regarding adsorbed protein orientation. The absorbance bands in the near-UV wavelength range (260–300 nm), which are accessible with CD, are sensitive to the microenvironment around disulfide bonds and aromatic residues, which are often found in both solvent accessible and inaccessible locations throughout protein. This can be potentially useful for detecting changes in protein tertiary structure or the binding of ligands to proteins since changes in the microenvironment around these residues cause shifts in the absorbance bands.^{24, 25} However, this information is purely qualitative and thus of limited usefulness.

The objective of this research project was to conduct side-chain modification studies to complement CD spectroscopy and bioactivity assays of adsorbed enzyme layers in order to provide a greater level of detail of the adsorption-induced changes to tertiary structure and orientation of these adsorbed enzymes. These methods were applied to study the effect of adsorption on bioactivity of two enzymes (hen egg-white lysozyme, HEWL; and glucose oxidase, GOx) on alkanethiol self-assembled monolayers (SAMs). The enzymes were nonspecifically and noncovalently bound onto planar, gold-coated glass (SiO₂) substrates that were surface modified using alkanethiols with four different functional groups (CH₃, OH, NH₂, and COOH) to investigate adsorption onto hydrophobic, hydrophilic, positively-charged, and negatively-charged surfaces respectively. The selective modification of amino acids has seen widespread use for more than 40 years in the study of protein structure and function because of its simplicity and effectiveness²⁶ and has recently been applied to probe the effect of adsorption on the structure of fibrinogen by Scott and Elbert by the modification of lysine residues.²⁷ Tryptophan (Trp) is an ideal residue to label for our purposes since solvent accessible Trps are located near the active site of both HEWL and GOx as well as within their normally solvent-inaccessible hydrophobic core. Solvent-accessible Trps can be covalently modified under mild conditions and physiological pH using dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS; 294.2 g/mol) and the extent of modification can be determined spectrophotometrically in basic solutions (pH 10).²⁸ This allows for quantitative information to be obtained regarding the degree of changes to the enzyme's tertiary structure and/or its orientation post-adsorption. An increased presence of modified Trps in the adsorbed enzymes following DHNBS treatment compared to those in solution provides definitive evidence that previously solvent-inaccessible residues are being exposed upon adsorption, thus indicating the occurrence of adsorption-induced unfolding of the tertiary structure, whereas a decreased presence of

modified Trps indicates regions of the enzyme that are sterically blocked by either the surface or neighboring adsorbed enzymes, thus providing information regarding adsorbed enzyme orientation. When correlated with changes in secondary structure, as measured by CD, and the changes in bioactivity following adsorption, this combination of assays provides insight into whether changes in an enzyme's bioactivity are due to adsorbed orientation, adsorption-induced conformational changes, or a combination of both.

Experimental Methods

Model Enzymes

The enzymes used in this study, as well as all buffers and reagents, were purchased from Sigma unless otherwise stated. Stock enzyme solutions (5.0 mg/mL) were prepared by suspending lysozyme (HEWL) derived from hen egg whites (14.4 kDa, pI = 11.0, PDB# 1GXV) and glucose oxidase (GOx), in its dimeric, glucosylated form, derived from *aspergillus niger* (160 kDa, pI = 4.2, PDB# 1CF3) in a 10 mM potassium phosphate buffer. The buffer was adjusted to a pH of 7.4 by mixing the appropriate amounts of 10 mM K_2HPO_4 and KH_2PO_4 solutions.

Alkanethiol SAMs

Bare quartz slides (Chemglass, Vineland, NJ) were coated with a 30 Å chromium adhesion layer followed by a 100 Å gold layer using a thermal vapor deposition (TVD) evaporator (Model E 12 E, Edwards High Vacuum Ltd.). The slides were thoroughly cleaned, prior to coating, by incubating them for 30 minutes in each of the following solutions (50°C), in order, then repeating the process a second time: "piranha" wash (7:3 H_2SO_4/H_2O_2), a basic solution (1:1:5 $NH_4OH/H_2O_2/H_2O$), and an acidic solution (1:1:5 $HCl/H_2O_2/H_2O$). The slides were thoroughly rinsed with nanopure water after each step then dried with flowing nitrogen gas and placed in the evaporator for deposition. After coating, the slides were then sonicated at room temperature for 1 min in a sulfuric acid solution (8:2 H_2SO_4/H_2O_2), rinsed with nanopure water followed by 100% ethanol, and placed into the appropriate 1.0 mM alkanethiol solution (Aldrich, Asemblon, and Prochimia) in ethanol for a minimum of 16 hours to form the SAM surfaces. All of the alkanethiols ($HS-(CH_2)_n-R$) used in these experiments have an 11-carbon alkyl chain and one of the following terminal groups: R = CH_3 , OH, NH_2 , or COOH.

Prior to surface characterization and protein adsorption, all SAMs were cleaned to remove any hydrocarbon contaminants that may have adsorbed onto their surface. Methyl SAMs were rinsed with ethanol, hexane, ethanol, nanopure water, and then placed in a 10 mM phosphate buffer. The non-methyl SAMs were rinsed with a 0.005% (v/v) Triton® X-100 solution, nanopure water, ethanol, acetone, nanopure water, and then placed in the phosphate buffer.

Ellipsometry

Thickness measurements were taken on a Sopra GES 5 variable-angle spectroscopic ellipsometer prior to surface modification and after surface treatment at six different spots to establish the optical constants of the metallic deposition layers and to calculate the thickness of the SAMs. The spectra were collected at an incidence of 70° in the wavelength range of 250–800 nm at 10 nm intervals. The layer thickness was calculated using the regression method in Sopra's Winelli (ver. 4.07) software.

Contact Angle Goniometry

The surface energy of the gold and the SAM surfaces were characterized by contact angle goniometry using a CAM 200 optical contact-angle goniometer from KSV Instruments, Ltd.

The advancing contact angles from six separate drops of nanopure water (pH 7) were measured on each of the sensor chips before and after surface modification.

X-Ray Photoelectron Spectroscopy

The SAMs were characterized by X-ray photoelectron spectroscopy (XPS) to obtain the chemical composition of the monolayers and ensure purity. The bare and surface treated gold slides were dried and packaged in a nitrogen environment and sent to NECSAC/BIO at the University of Washington for all XPS analysis. Surface analysis was conducted using a Surface Science Instrument (SSI) X-Probe spectrometer (Mountain View, CA) or a Kratos-Axis Ultra DLD spectrometer, equipped with a monochromatic Al K α source (KE = 1486.6 eV), a hemispherical analyzer and a multichannel detector. Spectra were collected at a photoelectron takeoff angle of 55° and at 80 eV for survey spectra and 20 eV for high resolution C1s and S2p spectra. Elemental compositions were determined from the peak areas in the spectra, using the SSI data analysis software or Kratos Vision 2 software program.

Circular Dichroism

CD spectra were collected using a Jasco J-810 spectropolarimeter over the wavelength range of 190–300 nm using a water-cooled sample holder (Jasco) attached to a circulating water bath operating at 15°C to reduce buffer evaporation. The structural contents of the enzymes were measured using 1.0 mg/mL enzyme solutions in a 10 mM phosphate buffer (pH ~ 7.4) loaded into 0.10 mm path length demountable quartz cuvettes (Starna). Background spectra were collected of the pure buffer for background correction prior to the analysis of enzyme solutions. In previous studies performed by our group, the presence of an adsorbed layer of protein on the surfaces of the quartz cuvette, which is unavoidable, was shown to have a negligible effect on the measured solution structure of the protein.²⁹

Surface modified, gold-coated quartz slides replaced the flat quartz window of the demountable cuvette for adsorption studies. A custom cuvette holder (Figure 1) was designed for these studies so that four adsorbed monolayers of the enzyme could be scanned simultaneously, which was necessary to provide sufficient CD signal-to-noise ratio for analysis. Background spectra were collected for each set of four slides in pure buffer prior to enzyme adsorption (4 sets per SAM composition). After the spectra were collected, the slides were immersed in a dish filled with buffer then the appropriate amount of the enzyme stock solution was pipetted into the dish to yield an enzyme concentration of 1.0 mg/mL. The slides were incubated in the enzyme solutions at room temperature for 24 hours to allow the enzyme to fully saturate the surface. Any residual, unbound enzyme was removed by diluting with flowing nanopure water for 5 minutes. This also prevented the slides from being exposed to any denatured proteins at the air/water interface when they were removed from the dish. The slides were transferred into a clean dish filled with buffer, mounted to the other half of the demountable cuvette under buffer, and then placed in the cuvette holder.

The following equations were used to convert the background corrected spectra from raw ellipticity (Θ , mdeg), provided by the instrument, to molar ellipticity ($[\Theta]$, deg·cm²/dmol):³⁰

$$[\Theta] = (\Theta \times M_0) / (10,000 \times C_{\text{soln}} \times L) \quad (1)$$

$$[\Theta] = (\Theta \times M_0) / (10,000 \times C_{\text{ads}}) \quad (2)$$

where M_0 is the mean residue molecular weight (118 g/mol), C_{soln} is the enzyme concentration in solution (g/mL), C_{ads} is the enzyme concentration on the SAM surface (g/cm²), and L is the path length through buffer (cm).

The enzymes concentrations in solution and on the surface, C_{soln} and C_{ads} , respectively, were determined using the peptide absorbance peak at 195 nm (A_{195}). Calibration curves were constructed for each enzyme in solution by plotting A_{195} vs. the enzyme concentration multiplied by the path length ($C_{\text{soln}} \times L$) for serial dilutions of the enzyme stock solutions. The concentrations of the stock solutions were verified by bicinchoninic acid (BCA) assays (Pierce) to ensure the accuracy of the calibration curves. The Beer's Law relationship, which is expressed as:^{31, 32}

$$A_{195} = \epsilon_{\text{soln}} \times C_{\text{soln}} \times L \quad (3)$$

is used to relate A_{195} to both C_{soln} and C_{ads} , with C_{ads} being obtained by replacing $C_{\text{soln}} \times L$ in Eqn. (3) with C_{ads} . It should be noted that both $C_{\text{soln}} \times L$ and C_{ads} represent the total amount of protein per unit area that the light beam passes through during a CD measurement. The slope of each calibration curve is the extinction coefficient (ϵ_{soln} , with units of mL/(g·cm) or cm²/g), which was used to calculate C_{soln} and C_{ads} from:

$$C_{\text{soln}} = \frac{A_{195}}{\epsilon_{\text{soln}} L} \text{ and } C_{\text{ads}} = \frac{A_{195}}{\epsilon_{\text{soln}}} \quad (4)$$

The validity of the use of spectrophotometry to measure C_{ads} via Eqn. (4) has been verified by ellipsometry, as reported in one of our previous publications.³³

The relationships expressed in Eqn. (4) can be plugged into their respective relationships shown in Eqns. (1) and (2) to convert the spectra into units of molar ellipticity. The spectra were then deconvoluted using CDPro software to generate a quantitative assessment of the percentage of the different secondary structures (i.e., α -helix and/or β -sheet) in the enzyme solution and after adsorption onto the SAM-surfaces.^{34, 35} With respect to helicity, the deconvolution algorithm used reports the total helical content (i.e., total percentage of α -helix and 3_{10} -helix combined).

Bioactivity Assays

The custom designed cuvette used for bioactivity assays is illustrated in Figure 2. The cuvette was designed to hold 4 gold coated slides while allowing for the transmission of light in the visible wavelength range for colorimetric activity assays and the far UV range to determine C_{ads} . The benefit of using this cuvette is that it has a high surface to volume ratio, it requires only 400 μ L of the substrate to fill the cuvette, and it provides ease for pipetting solutions in and out of the cuvette. This holder has also been used in previous CD studies;³³ however, the setup shown in Figure 1 was used for CD in this study due to the shorter path length through buffer, which provides a higher signal-to-noise ratio at a given scanning speed, thus aiding in the determination of the adsorbed structure of a small enzyme like lysozyme.

The bioactivities of the adsorbed enzyme layers were assayed after the completion of CD analysis. The amount of adsorbed enzyme was quantified both before and after the bioactivity assays were run to ensure that the bioactivity assays did not cause any of the protein to be desorbed from the surface. After initial protein quantification, the bioactivity was assayed by replacing the buffer with 400 μ L of the appropriate substrate solution: 3 mg/L peptidoglycan from *Staphylococcus aureus* dyed with Remazol Brilliant Blue R (RBB-R) read at 595 nm³⁶ for HEWL and 2.4 mL of 0.66 mg/mL o-dianisidine, 0.5 mL of 10% (w/v) -D-glucose, and 0.1 mL of 5 mg/mL horseradish peroxidase (HRP) read at 540 nm for GOx.³⁷ For HEWL, low molecular weight fragments are released as the enzyme digests the peptidoglycan. The slides were incubation in the substrate for 2 minutes and then 200 μ L of the substrate was pipetted from the cuvette and added to 600 μ L of ethanol to terminate the

reaction and precipitate the undigested, high molecular weight fragments. The peptidoglycan suspension was vortexed and allowed to equilibrate for 10 minutes then centrifuged at 1,500 g for 10 minutes. Afterwards, the supernatant was then collected and the absorbance read at 595 nm. To avoid potential complications from competitive adsorption between GOx and HRP,³⁸ the slides were incubated in the o-dianisidine/ -D-glucose mixture for 2 minutes, without the addition of HRP, then 290 μ L of the substrate was pipetted out of the cuvette and into a 48 well plate. 10 μ L of the HRP solution was then added to the well and the solution was aspirated several times before recording the absorbance.

The specific activities of the adsorbed layers were calculated by normalizing the measured rates of change in absorbencies by the concentration of the protein on the surface (C_{ads}). Controls were performed using a series of enzyme concentrations (1.0–100 μ g/mL) to ensure that the specific activity of the enzymes were constant over the range and not diffusion-limited. The specific activity of each adsorbed enzyme layer was normalized by its specific activity in solution and multiplied by 100% to determine the relative activities.

Tryptophan Modification in Solution

To determine the change in the number of Trps that were accessible to labeling before and after enzyme adsorption, baseline studies were first conducted to modify the solvent accessible Trps of the enzymes in their native state in solution based on well-established protocols.^{39, 40} These experiments were performed using 2.0 mg/mL enzyme solutions prepared in the phosphate buffer. The proteins, as well as their buffers and substrates, were purchased from Sigma unless stated otherwise. Fresh DHNBS solutions were prepared in buffer at concentrations that were 100 times the molarity of the enzyme solutions. Equal aliquots of the enzyme and DHNBS solutions were added together, vortexed for 2 min, and allowed to react for 30 minutes in the dark. The solutions were then dialyzed overnight in flowing nanopure water to remove excess reagent. Samples were set aside at this time for protease digestion and peptide mapping by electrospray ionization mass spectrometry (ESI-MS) at the Proteomics Facility, Clemson University Genomics Institute (CUGI) to determine the occupancy of modified Trp residues. The concentration of DHNBS was calculated by mixing equal aliquots of the dialyzed solution and a 1.0 mM NaOH solution to increase the pH above 10 so that the absorbance peak at 410 nm could be read. The protein concentration was measured using a bicinchoninic acid (BCA) assay kit (Pierce) and the DHNBS concentration was divided by the protein concentration to yield the average number of modified Trps per molecule.

Protease Digestion

To prepare the modified enzyme samples for ESI-MS, in-liquid protease digestions were performed according to previously reported methods.^{41, 42} Briefly, 4.0 μ L of each protein solution (~ 1.0 mg/mL unmodified controls and DHNBS modified test samples) was added to 100 μ L of 1.0 mM ammonium bicarbonate. To reduce the disulfide bonds and terminate the reaction between DHNBS and Trp, 3.0 μ L of 45 mM dithiothreitol (DTT) was added and the sample was incubated at 37°C for 20 minutes. After cooling to room temperature, the reduced cysteines were alkylated by adding 4.0 μ L of 100 mM iodoacetamide (IAA) and reacting in the dark for 20 minutes. The excess reagents were removed by lyophilizing to completion in a Savant SpeedVac (Savant Instruments Inc., Holbrook, NY) for 1 hour.

HEWL samples were digested by trypsin and GOx samples were digested by trypsin, chymotrypsin, and Glu-C at protease to substrate ratio of 1:50 (w/w) using 0.04 μ g/ μ L protease solutions in their respective buffers. For tryptic digestion, sequence-grade porcine trypsin (5,000 units/mg) (Promega) was diluted with 10 mM HCl and digestion was carried out at 37°C for 18 hours. A 50 mM ammonium bicarbonate buffer was used for Glu-C and

samples were incubated overnight at room temperature for digestion. Chymotrypsin was diluted in 100 mM ammonium bicarbonate and incubated at 37°C for over 18 hours. Following incubation, 1.5 μ L of 0.1% (v/v) trifluoroacetic acid (TFA) was added to stop the digestion. The resulting peptide solutions were then lyophilized to completion for 1 hour.

Mass Spectrometry

Lyophilized peptide samples were suspended in 50 μ L of an injection solution containing 50% methanol and 0.1% formic acid (FA), and then vortexed and centrifuged at 16,000 g for 3 min to remove any particles in the samples. 2.0 μ L of the supernatant was injected into a CapLC system using a capillary autosampler and peptides were separated by reversed-phase high performance liquid chromatography (HPLC) using a NanoEase C₁₈ column (Waters Corporation, Milford, MA) over a 2–40% acetonitrile (ACN) gradient against water over 60 min at 7.0 μ L/min flow rate in the presence of 0.1% FA. Reversed-phase separation was followed by mass scan acquisition of the peptide m/z value, retention time, and intensity using the quadrupole time-of-flight (Q-ToF) micro tandem mass spectrometer (Waters Corporation, Milford, MA).

The total ion current (TIC) chromatograms were acquired, in triplicate, over duration of 30 minutes for each sample. The chromatograms were filtered to remove noise and the peaks were smoothed, centered, and deconvoluted to generate a singly charged peak list using the MaxEnt 3 algorithm (Waters Corporation, Milford, MA). The peak lists were entered into the General Protein Mass Analysis for Windows software (ver. 6.11, ChemSW, Inc., Denmark) for peptide mapping. The software checked the peak lists against the theoretical peaks generated by fragmenting the primary sequence at the known cleavage sites of the protease used for digestion and considered the possible modification of Trp residues by DHNBS. Peptide peaks from the unmodified samples that were identified as DHNBS modified were deemed false positives and removed from modified peak lists.

Tryptophan Modification for Adsorbed Enzymes

The cleaned, alkanethiol treated slides were placed into dishes filled with a sufficient amount of phosphate buffer so that they were completely submerged. The appropriate amount of a protein stock solution (10 mg/mL) was then pipetted into the dishes so that the final concentration was 1.0 mg/mL. After 24 hours of incubation at room temperature, the dishes were infinitely diluted under running distilled water for 5 minutes and the slides were rinsed thoroughly with nanopure water. The protein concentration on the surface was determined by measuring the peptide absorbance peak at 195 nm using a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD), using the same custom cuvette and procedures used for CD studies conducted by our group.²⁹ The slides were transferred from the cuvette into a dish filled with a 0.5 mM DHNBS solution and allowed to react for 30 minutes in the dark. Again the dishes were infinitely diluted under running water and the slides rinsed with nanopure water before being transferred into the cuvette filled with a 0.5 mM NaOH solution. The DHNBS concentration in the adsorbed protein layer was calculated by measuring the absorbance peak at 410 nm.

For enzymatic digestion of the modified, adsorbed HEWL layers, the surfaces with the adsorbed enzymes were placed in the PEEK holder (Figure 2), which was filled with 400 μ L of a 1.0 mM ammonium bicarbonate solution. The adsorbed HEWL layers were reduced and alkylated prior to digestion using methods similar to those applied for the enzymes in solution and excess reagents were removed by lyophilizing overnight. Trypsin suspended in a 10 mM HCl solution was then added to the PEEK holder and digestion was carried out at 37°C for 18 hours. The ratio of the concentration of trypsin to the surface concentration of HEWL was 1:50. The solutions containing the peptide fragments were collected after

digestion, lyophilized, and processed according to the methods described in the mass spectrometry section.

Statistical Analysis

The mean and 95% confidence interval (CI) were calculated for all sets experimental data collected. Statistical differences were determined using a Student's unpaired t-test with values of $p < 0.05$ considered to be statistically significant.

Results and Discussion

Surface Characterization

The SAMs were characterized using contact angle goniometry and ellipsometry to obtain the advancing contact angle, using a 10 mM phosphate buffer (pH 7.4), and the thickness of the alkanethiol layer on the gold surface (Table 1). The contact angle values on the SAMs were in close agreement with other published values.^{43,44} Ellipsometry measurements confirmed that the films consisted of a single monolayer on each surface. The XPS results (Table 1) also verified the presence of a single alkanethiol monolayer with negligible amounts of oxidized sulfur or contaminants. The presence of oxygen on the NH_2 -SAM and an excess of oxygen on the OH-SAM are believed to be due to tightly bound water on the surface.⁴⁵

Previously, we measured the surface dissociation constant (pK_a) of the COOH - and NH_2 -SAMs using SPR spectroscopy.⁴⁶ It was determined that the pK_a based on the pH of the bulk solution far away from the surface was 7.4 ± 0.2 and 6.5 ± 0.3 ($N=4$, mean $\pm$ 95% CI) for the COOH - and NH_2 -SAMs respectively. Therefore, when the bulk pH is 7.4 approximately 50% of the COOH groups should be deprotonated (negatively charged) and 11% of the NH_2 groups are protonated (positively charged). It should be noted that the charged surfaces will create a concentration gradient of ions near the surface resulting in a pH shift at the surface due to the excess of H^+ or OH^- ions. Based on the analytical model we presented,⁴⁶ we estimate that the pH at the surface of the COOH -SAM is actually 5.0 and 8.9 at the surface of the NH_2 -SAM, with these values being in line with what is expected for these functional groups.

Tryptophan Accessibility in Native Structures

Peptide fragments from the ESI-MS analyses were identified that covered all Trps in the two enzyme sequences. Table 2 lists the Trps that were identified in a peptide fragment as being modified and those that were unlabeled in all fragments containing that residue. Four out of HEWL's six Trp residues were identified as labeled with the two Trps in the hydrophobic core being unlabeled, thus identified as being solvent inaccessible (Figure 3), yet the average number of modified Trp per protein molecule, determined spectrophotometrically, was only 2.1 ± 0.3 . Therefore, all four Trps deemed solvent accessible are not being labeled in every enzyme but the ratio was highly reproducible and useful for comparison with the values determined from the adsorption studies. Likewise, six out of the ten Trps in the GOx subunit were modified by DHNBS (Figure 3) and the average number of modified Trps per subunit was determined to be only 1.3 ± 0.3 , but again reproducible in all samples.

Adsorbed Lysozyme Layers

Figure 4.A presents the extent of Trp modification (left-hand axis) and percent reduction in helix content (right-hand axis) for each type of SAM surface. As indicated, the average number of solvent accessible Trps per HEWL molecule was significantly decreased with respect to HEWL in solution after adsorption onto each of our four functionalized SAM surfaces. Adsorption on the CH_3 , OH, and NH_2 -SAMs resulted in a significant loss in its helical content, while the structure of lysozyme was not significantly different from solution

on the COOH-SAM. There was no statistical difference in the loss of helices between the CH₃-, OH-, and NH₂-SAMs (i.e., compared to each other) yet there was a wide range in the average number of labeled Trps per molecule between these surfaces. Thus, as shown in Figure 4.B, there is a very low correlation between Trp modification and the reduction in helical structure. These results therefore suggest that conformational changes had a minimal effect on the change in Trp solvent accessibility following adsorption.

The solvent accessibility of Trps in HEWL is plotted in Figure 5.A (left-hand axis) along with its relative activity (right-hand axis). As clearly indicated, both the Trp accessibility and activity of the adsorbed enzyme layers decreased after adsorption onto each of the alkanethiol SAMs. Due to the location of HEWL's Trps with respect to its active site, the reduction in both the accessibility and bioactivity suggests that adsorbed orientation is the dominate factor in the loss of activity post-adsorption. As shown in Figure 3, there are two solvent accessible Trps in the active cleft of HEWL and the other two are located within the same face, adjacent to the cleft. Thus it is reasonable to assume that the accessibility of DHNBS to modify these Trps should be similar to the accessibility of the substrate to the bioactive cleft of this enzyme, and a strong correlation between the Trp accessibility and activity is clearly shown in Figure 5.B. The two SAM surfaces where HEWL exhibited the lowest bioactivities, the CH₃- and COOH-SAMs, also had the lowest amounts of accessible Trps. Both of these values (i.e., the number of labeled Trps and enzyme activity) were significantly higher on the other two SAMs. The disparity in these values between the charged surfaces (i.e., the NH₂-SAM vs. COOH-SAM surfaces) was anticipated since the HEWL carries a large net positive charge at physiological pH and the face that is attracted on one surface should be repelled on the other. Although there are negatively charged residues within HEWL's active cleft, the regions on both sides of its active cleft are positively charged, thus likely causing the enzyme to adsorb with its bioactive cleft facing towards the surface with subsequent reduced activity on the COOH-SAM.⁴⁷

The initial distribution of orientations on the neutral SAMs (i.e., the OH-SAM and CH₃-SAM surfaces) is expected to be generally similar since there should be no electrostatic interactions between the protein and the surface to orient the protein as it adsorbs. However, HEWL does have a concentration of nonpolar amino acids, including several Trp residues, along its bioactive cleft, which can be expected to cause preferential adsorption of that face to the CH₃-SAM surface. As shown in Figure 5.A, there were an average of 1.5 ± 0.3 modified Trps per protein molecule adsorbed on the hydrophilic OH-SAM and only 0.1 ± 0.04 on the hydrophobic CH₃-SAM. This 10-fold difference indicates that there were substantial differences in the solvent accessibility of the Trps in the adsorbed HEWL layers between these two surfaces, with those on the CH₃-SAM largely being obstructed by the surface or neighboring HEWL molecules. The relatively large loss in bioactivity on the CH₃-SAM compared to the OH-SAM also indicates that the accessibility of the active cleft is drastically reduced on the hydrophobic surface. When taken in combination with the lack of a statistical difference in the secondary structure of the adsorbed HEWL layers on the CH₃- and OH-SAMs (Figure 4), these results support that the difference in activity was caused by a net difference in the orientation of adsorbed HEWL on these two surfaces.

To provide further support that the loss in activity on the CH₃-SAM was due to a shift in the distribution of orientations with respect to the OH-SAM, the modified HEWL layers on these surfaces were enzymatically digested and sequenced to specifically locate the positions of the solvent accessible Trps. Peptide fragments were identified in the digestions of the adsorbed layers on both SAMs that contain all six Trps (70% sequence coverage achieved for every sample). Figure 6 shows a portion of the MS scans from the unmodified control and the peptide digest from HEWL adsorbed on OH-SAMs and DHNBS modified and highlights the 153 Da increase in mass due to Trp modification. The only Trp that was

modified on the CH₃-SAM was Trp123, which is located on the end opposite the active cleft (Figure 7), whereas five were modified on the OH-SAM, including the two in the active cleft. The highly exposed Trps within the active cleft play an important role in catalysis by holding ligands in place via hydrophobic interactions during cleavage.^{48–50} The steric blockage of these two residues does not necessarily inactivate the enzyme but their lack of solvent accessibility, as indicated by their unlabeled state following adsorption, suggests that access to the active site is limited in the molecules throughout the adsorbed layer on the CH₃-SAM. It should also be noted that Trp108 was unlabeled in the control experiments but labeled after adsorption on the OH-SAM, highlighting that significant changes in tertiary structure occur even on the hydrophilic surface that could also play a role in the activity of the adsorbed layers.

Adsorbed Glucose Oxidase Layers

In distinct contrast to HEWL, after the adsorption of GOx to each of the SAM surfaces there was a substantial increase in the Trp accessibility of the adsorbed layers with respect to GOx in its native state. This is a clear indication of adsorption-induced unfolding of the tertiary structure of the enzyme leading to the exposure of previously solvent-inaccessible Trps. The most significant changes occurred on the more hydrophobic surfaces (i.e., the CH₃- and NH₂-SAMs; see Table 1), which was expected due to GOx's size, lack of disulfide bonds, and the increased tendency of proteins to unfold on a hydrophobic surface.^{1, 51, 52}

As shown in Figure 8, there is a strong correlation between the solvent accessibility of Trp and the adsorption-induced loss of α -helix. The number of solvent accessible Trps more than doubled after the adsorption of GOx on all four SAMs compared to the solution structure, signifying that previously buried residues were being exposed on all surfaces. The two hydrophobic surfaces, the CH₃- and NH₂- SAMs, induced the highest degree of destabilization to helices and also exposed the most Trps upon adsorption. Conversely, adsorption-induced changes to conformation and Trp accessibility were significantly lower on the OH- and COOH-SAMs.

However, unlike HEWL, the change in the solvent accessibility of Trp residues in adsorbed GOx on all four of the SAM surface did not correlate well with the changes in bioactivity (Figure 9). This indicates that the changes in adsorbed activity on these four surfaces were not due to conformational effects alone, with GOx activity also being influenced by adsorbed orientation. Considering first the neutral SAMs (OH-SAM and CH₃-SAM), the number of modified residues and the loss in helicity were significantly higher on the CH₃-SAM, as expected, yet surprisingly the adsorbed GOx layer was more active on this surface than any other surface. Since strong orientational differences for GOx are not expected on the neutral SAMs due to a lack of electrostatic interactions, we have no basis to assume that adsorbed orientation played a larger role in influencing the enzyme's adsorbed-state activity on one of these surfaces compared to the other. We therefore propose that the higher activity of GOx on the CH₃- SAM than the OH-SAM is due to the observed conformational differences, with the unfolding of GOx on the hydrophobic surface causing increased accessibility of the active pocket, thus facilitating the transport of the substrate and its reaction products in and out of the pocket, respectively. In contrast, on the OH-SAM, the pocket remains much closer to its native-state conformation and we propose that adsorbed orientation restricts access to the relatively deep active pocket due to steric blocking by the surface and/or neighboring adsorbed enzymes for a substantial fraction of the adsorbed protein molecules, thus substantially retarding the net bioactivity of the adsorbed enzyme layer.

Considering the charged SAM surfaces, Figure 8 shows that GOx underwent a greater degree of unfolding on the NH₂-SAM compared to the COOH-SAM as indicated by the

greater degree of Trp modification and greater loss in helicity on the NH₂-SAM surface. If unfolding-induced opening of the active pocket was the only mechanism controlling the activity of GOx on our set of four SAM surfaces, then the adsorbed GOx layer on the NH₂-SAM would be expected to have a higher activity than the layer on the COOH-SAM. However, the data shows that the opposite effect occurred on the charged surfaces, with the activity of adsorbed GOx being twice as high on the COOH-SAM as on the NH₂-SAM surface. These results thus suggest that in this case electrostatically driven orientational effects played a more dominant role in influencing the enzyme's adsorbed-state activity.

From these combined results, we propose that the activity of adsorbed GOx can be enhanced by either favorably orienting the enzyme over a negatively charged surface, or by partially unfolding the enzyme on a hydrophobic surface, with each of these mechanisms enhancing access of the substrate to the active site of the adsorbed enzyme.

Conclusions

The methods presented in this study were developed for the purpose of providing a more comprehensive and synergistic set of protocols for assessing the orientation, the secondary and tertiary structures, and the bioactivity of adsorbed enzyme layers. While CD spectroscopy and bioactivity assays alone provide a wealth of information about the secondary structure and functionality of adsorbed enzyme layers, they provide almost no information about the tertiary structure and the delineation between changes in bioactivity due to conformational versus orientational effects. Side-chain specific chemical modification proved to be particularly useful when combined with the CD and the bioactivity results to provide greater insights regarding the influence of adsorption on the orientation and conformation of the enzymes, and the subsequent effects on their adsorbed-state bioactivity. The combination of these three techniques showed that the adsorption-induced changes in the activity of HEWL were primarily due to adsorbed orientation, whereas activity changes in GOx were due to a surface-dependent combination of both orientational and conformational effects.

The benefit of side-chain specific chemical modification is the versatility of the technique. The proteomics field has established an array of protocols over the past several decades for several different types of amino acid residues that can be modified individually or in conjunction with one another to gain insights about the structure and orientation of adsorbed proteins. Since these modifications are amino acid specific, they are protein independent and can be applied to any protein containing the residue(s) of interest. Thus, this technique can be readily used along with CD spectroscopy for the analysis of not only adsorbed enzymes, but adsorbed proteins in general. The developed techniques thus present a valuable set of methods that can be used to investigate proteins in their adsorbed state for any application where protein-surface interactions are of critical importance.

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Figure 1. Customized cuvette holder used for CD analysis of adsorbed protein layers of surface treated, gold-coated quartz slides. The top view represents the top of an individual demountable cuvette.

Figure 2.

Top view of custom cuvette designed for the Jasco spectropolarimeter for operation in the wavelength range of 190 to 590 nm for protein quantification and spectrophotometric bioactivity assays. The outer wall of the cuvette was made from polyetheretherketone (PEEK).

Figure 3.

Protein data bank (PDB) images of HEWL (PDB #1GXV) and a subunit of GOx (PDB #1CF3). The green residues are the Trps that were modified by DHNBS after reacting 1.0 mg/mL protein solutions at a 50× molar excess of the reagent. The grey residues represent Trps that were not modified by the reagent.

(A)

(B)

Figure 4.

(A) Left Column (left y-axis): The number of solvent accessible Trps per HEWL molecule in solution and after adsorption. Right Column (right y-axis): The percent loss of helices after adsorption with respect to the measured solution structure (% reduction = $100\% (\%_{\text{soln}} - \%_{\text{ads}}) / \%_{\text{soln}}$). (B) Correlation between the reduction in helix and the average number of modified Trps of the adsorbed HEWL layers. (N=4, mean $\pm$ 95% CI)

(A)

(B)

Figure 5.

(A) Left Column (left y-axis): The average number of solvent accessible Trps per HEWL molecule in solution and after adsorption. Right Column (right y-axis): The relative activities of the adsorbed HEWL layers. (B) Correlation between the relative activity and the average number of modified Trps of the adsorbed HEWL layers. (N=4, mean $\pm$ 95% CI)

Figure 6.

(A) Deconvoluted MS scan of tryptically digested HEWL after adsorption on OH-SAMs and DHNBS modification. Arrows indicate fragments where either Trp108 or Trp111 is modified. (B) Scan of the unmodified HEWL control with arrows indicating the corresponding unmodified peptides.

Figure 7.
PDB images of HEWL (PDB #1GXV). The grey residues indicate Trps that were detected as unlabeled after adsorption onto CH₃- and OH-SAMs followed by DHNBS modification and tryptic digestion. The green residues represent Trps that were modified during the process.

(A)

(B)

Figure 8.

(A) Left Column (left y-axis): The number of solvent accessible Trps per subunit of GOx in solution and after adsorption. Right Column (right y-axis): The percent loss of helices after adsorption with respect to the measured solution structure ($\% \text{ reduction} = 100\% (\%_{\text{soln}} - \%_{\text{ads}}) / \%_{\text{ads}}$). (B) Correlation between the loss of helix and the average number of modified Trps of the adsorbed GOx layers. (N=4, mean $\pm$ 95% CI)

(A)

(B)

Figure 9.

(A) Left Column (left-hand y-axis): The number of solvent accessible Trps per GOx molecule after in solution and after adsorption. Right Column (right-hand y-axis): The relative activities of the adsorbed GOx layers. (B) Correlation between the relative activity and the average number of modified Trps of the adsorbed GOx layers. (N=4, mean $\pm$ 95% CI)

Table 1

Atomic composition (%) of the surface-treated gold slides, with the exception of Au, as measured by XPS (N=3, mean $\pm$ 95% CI). Advancing contact angle (10 mM phosphate buffer solution at pH 7.4) and the ellipsometry measurements of the SAMs (N=6, mean $\pm$ 95% CI).

Alkanethiol	C _{1s}	S _{2p}	N _{1s}	O _{1s}	Contact Angle (°)	Thickness (Å)
HS-(CH ₂) ₁₁ -NH ₂	85.3 $\pm$ 1.4	3.2 $\pm$ 0.4	6.3 $\pm$ 0.5	5.2 $\pm$ 0.5	47.6 $\pm$ 1.8	14.7 $\pm$ 2.5
HS-(CH ₂) ₁₁ -COOH	83.8 $\pm$ 3.2	2.8 $\pm$ 0.2	- -	13.4 $\pm$ 0.6	17.9 $\pm$ 1.3	15.8 $\pm$ 1.9
HS-(CH ₂) ₁₁ -CH ₃	95.9 $\pm$ 1.0	4.1 $\pm$ 0.3	- -	- -	100.9 $\pm$ 1.9	11.5 $\pm$ 2.2
HS-(CH ₂) ₁₁ -OH	84.6 $\pm$ 1.2	4.2 $\pm$ 0.8	- -	11.2 $\pm$ 0.3	17.6 $\pm$ 1.9	12.1 $\pm$ 1.4

Table 2

Results of Trp modification (as designated by amino acid primary sequence location) and protein sequencing of 1.0 mg/mL protein solutions reacted at a 50× molar excess of DHNBS. (N=4, mean $\pm$ 95% CI)

	HEWL	GOx
Labeled Trps	62, 63, 111, 123	111, 122, 350, 402, 426, 503
Unlabeled Trps	28, 108	131, 133, 232, 376
Average Number of Modified Trps per Enzyme Molecule	2.1 $\pm$ 0.3	1.3 $\pm$ 0.3 (per subunit)