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Probing the Orientation of Electrostatically Immobilized Protein G B1 by Time of Flight Secondary Ion Spectrometry, Sum Frequency Generation and Near-edge X-Ray Adsorption Fine Structure Spectroscopy

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Abstract

To fully develop techniques that provide an accurate description of protein structure at a surface, we must start with a relatively simple model system before moving on to increasingly complex systems. In this study, x-ray photoelectron spectroscopy (XPS), sum frequency generation spectroscopy (SFG), near-edge x-ray adsorption fine structure (NEXAFS) spectroscopy, and time-of-flight secondary ion mass spectrometry (ToF-SIMS) were used to probe the orientation of Protein G B1 (6 kDa) immobilized onto both amine (NH_3^+) and carboxyl (COO^-) functionalized gold. Previously, we have shown that we could successful control orientation of a similar Protein G fragment via a cysteine-maleimide bond. In this investigation, to induce opposite end-on orientations, a charge distribution was created within the Protein G B1 fragment by first substituting specific negatively charged amino acids with neutral amino acids and then immobilizing the protein onto two oppositely charged self-assembled monolayer (SAM) surfaces (NH_3^+ and COO^-). Protein coverage, on both surfaces, was monitored by the change in the atomic % N, as determined by XPS. Spectral features within the SFG spectra, acquired for the protein adsorbed onto a NH_3^+ -SAM surface, indicates that this electrostatic interaction does induce the protein to form an oriented monolayer on the SAM substrate. This corresponded to the polarization dependence of the spectral feature related to the NEXAFS N_{1s} to π^* transition of the β -sheet peptide bonds within the protein layer. ToF-SIMS data demonstrated a clear separation between the two samples based on the intensity differences of secondary ions stemming from amino acids located asymmetrically within Protein G B1 (Methionine: 62 and 105 m/z; Tyrosine: 107 and 137 m/z; Leucine: 86 m/z). For a more quantitative examination of orientation, we developed a ratio comparing the sum of the intensities of secondary-ions stemming from the amino acid residues at either end of the protein. The two-fold increase in this ratio, observed between the protein covered NH_3^+ and COO^- SAMs, indicates opposite orientations of the Protein G B1 fragment on the two different surfaces.

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1. Introduction

The ability to characterize the structure of a protein at a surface has important implications for the understanding of basic biological processes like cell signaling,^{1, 2} controlling protein adsorption on biomaterials,^{3, 4} and the construction of biomolecular sensors and microarray devices.^{5, 6} With the increased interest in the construction of biological based sensors, researchers have proposed a range of possible immobilization schemes based on coordination complexes^{7–10}, ligand-receptor,¹¹ covalent conjugation,^{10, 12–17} hydrophobic/hydrophilic driving forces^{18–20} and electrostatic interactions.^{21–23} The ability of these biological devices to bind specific targets is directly related to the accessibility of capture groups at the sensor surface.²⁴ For devices based on proteins and antibodies, these immobilization schemes must successfully orient binding sites so that they are accessible.

In parallel, as new protein immobilization platforms are built, a set of surface analytical tools that provide a clear quantitative view of protein structure, ordering and orientation at the sensor surface must also be developed. Earlier studies have demonstrated the promise of time-of-flight secondary ion mass spectrometry (ToF-SIMS),^{23, 25–28} sum frequency generation spectroscopy (SFG)^{29–33} and near-edge x-ray fine structure spectroscopy (NEXAFS)^{17, 34, 35} as a set of surface analytical techniques that provide complementary views of organic molecules at surfaces.

Taking advantage of ToF-SIMS' high mass resolution (>4000 m/m) and excellent surface sensitivity (~ 2 nm sampling depth) previous work mapped changes in conformation and orientation of relatively thick protein films (>10 nm).^{23, 26, 36–39} Recently Baugh *pet. al.* showed with a model system built upon a small covalently-immobilized protein (B1 domain of Protein G, 6 kDa), that they could determine differences in orientation by examining ratios of intensities of secondary ions originating from amino acid residues at opposite ends of the protein (C-terminus versus N-terminus).⁴⁰ Thus, ToF-SIMS can determine protein orientation even when the thickness of the protein is similar to the ToF-SIMS sampling depth. To complement the ToF-SIMS studies of protein orientation, Baugh *pet. al.* also used SFG and NEXAFS spectroscopy to examine the ordering of secondary structural elements within the protein.⁴⁰

In this investigation, we modified the model system previously built by Baugh *pet. al.*⁴⁰ and probed the orientation and ordering of a charge mutant of the Protein G B1 domain that was immobilized by electrostatic interactions. Here the protein immobilization was controlled by creating a charge distribution within the Protein G B1 domain by substituting negatively charged amino acids with neutral amino acids (i.e., mutating aspartic acid to asparagine). Negatively charged amino acids at the N-terminus were replaced with neutral amino acids by specific mutations, creating a dipole like distribution with a negatively charged region near the C-terminus (Figure 1). With this dipole two different orientations can be induced by electrostatic interactions between the protein and two oppositely charged surfaces (Figure 1). The orientation of secondary structures within this protein, at these charged substrates can then be assessed by both SFG and NEXAFS spectroscopy. Changes in overall end-on orientation will be described by ToF-SIMS, where intensity differences of specific secondary ions provide a method of discriminating between different protein orientations.

2. Experimental

2.1 Functionalized Substrates

1×1 cm² silicon substrates (Microelectronics Inc., San Jose, CA) were cleaned by sequential sonication in deionized water, dichloromethylene, acetone, and methanol. In a high vacuum electron beam evaporator (pressure $< 1 \times 10^{-6}$ Torr), substrates were then coated with a thin

layer of titanium (10 nm) followed by high purity gold (99.99%, 80 nm). Following established protocols,⁴¹ amine self-assembled monolayers (NH₂-SAMs) were assembled by submerging the Au coated substrates into a 0.5mM solution of 11-amino-1-undecanethiol, hydrochloride (Asemblon, Redmond, WA) in ethanol. The substrates were allowed to soak in this ethanol-thiol solution for at least 16 h. Following assembly, the substrates were removed from solution, rinsed with pure ethanol, and then vortexed within a 10% v/v NH₄OH ethanol solution. Carboxylic acid SAMs (COOH-SAMs) were assembled by soaking Au substrates in a 1-mM 11-mercaptoundecanoic acid (Aldrich, St. Louis, MO) ethanol solution for at least 16 h and then rinsed with pure ethanol. Following the rinse step, all substrates were dried and stored under nitrogen until analysis or protein adsorption.

2.2 Protein Mutagenesis, Expression and Purification

A mutant (D4'), with four charge-neutralizing mutations at one end of the Protein G B1 domain (E19Q, D22N, D46N, and D47N), was created in a synthetic Protein G B1 gene⁴² using the Quick Change protocol (Stratagene, La Jolla, CA), and confirmed by sequencing. Incorporation of a ¹³C-methionine label into the mutant was accomplished by expressing the protein in minimal media supplemented with 100 mg/L of labeled amino acid (L-methionine, methyl-¹³C; Cambridge Isotope Laboratories, Andover, MA) using the T7 expression system in BL21(DE3) *pE. coli* (Invitrogen, Carlsbad, CA). Expressed protein was isolated by thermal lysis and two steps of anion exchange chromatography as described previously⁴⁰ with one exception: 50 mM piperazine, pH 10.0, was used as the loading buffer in the second ion exchange step. Protein molecular weight and amino acid composition were confirmed using electrospray mass spectrometry and amino acid analysis (AAA Laboratory, Mercer Island, WA).

2.3 Protein Immobilization

Labeled D4' protein-buffer solutions, 0.5 mL of 1.5 mg/mL protein, were added to substrates in 24-well plates. For adsorption onto the NH₃⁺ and COO⁻-SAM substrates, a 20 mM bis-tris (pH 6.0) and a 20 mM sodium phosphate (pH 8.0) buffer were employed, respectively. The two different pH's were chosen to maximize the charge at the substrate⁴³ while maintaining both positive and negative charges within the protein. (The calculated pI for D4' $\cong$ 6.7) Binding proceeded for 4 h at room temperature. Following protein immobilization, samples were washed by serial dilution in buffer, followed by submerging samples in a series of stirred water solutions. Samples were then air-dried and stored under nitrogen.

2.4 X-ray photoelectron spectroscopy (XPS)

XPS compositions were determined from an average of three spots on three replicate samples. The data were acquired on a Kratos AXIS Ultra DLD instrument (Kratos, Manchester, England) in the hybrid mode using a 0° take-off angle (TOA) and a monochromatic Al K $\alpha_{1,2}$ x-ray source ($ph\nu = 1486.6$ eV). Here, the photoelectron TOA is defined as the angle between the surface normal and the axis of the analyzer lens. Atomic compositions were calculated from C_{1s} and Au_{4f} peak areas obtained from survey scans (0–1100 eV) plus O_{1s} (524–544 eV), N_{1s} (390–410 eV), and S_{2p} (155–173 eV) from narrow scans (analyzer pass energy = 80 eV for both survey and narrow scan spectra). Energy scales were calibrated by setting the large CH_p peak in the C_{1s} region to 285.0 eV and a linear background was subtracted for all peak area quantifications. The peak areas were normalized by the manufacturer supplied sensitivity factors and surface concentrations were calculated using CASA XPS (Casa Software Ltd).

The amount of protein on the surface was tracked by the nitrogen atomic percent (At %) determined from the N_{1s} signal. The NH₂-SAM substrates contain nitrogen, therefore, to determine just the contribution of the protein layer to the N_{1s} signal, contribution from the

underlying NH₂-SAM layer was removed. By examining the attenuation of the Au_{4f} signal from the Au substrate before and after protein immobilization the nitrogen contribution from just the protein layer can be calculated using Equation 1.^{10, 38}

$$N_{Norm} = N_p - N_s(Au_p/Au_s) \quad \text{Eqn 1}$$

Where, N_s and Au_s are the measured N and Au At %, respectively, from the NH₂ SAM covered Au substrate prior to protein immobilization; N_p and Au_p are the measured N and Au At %, respectively, from the NH₂ SAM covered Au substrate after protein immobilization; and N_{norm} is the N At % corresponding to just the protein layer.

2.5 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Positive secondary ion spectra were acquired on a TOF.SIMS 5–100 instrument (ION-TOF, Munster, Germany) using a pulsed 25 keV Bi₃⁺ primary ion beam under static conditions (primary ion dose < 10¹² ions/cm²). Spectra were collected from five 100 × 100 μm² regions per sample. Secondary ions were collected over a range of 0–400 m/z at a mass resolution (m/Δm) between 4000 and 8000. Spectra were mass calibrated using CH₂⁺, C₂H₂⁺, and AuSCH₂⁺ peaks. Mass calibration corrections were typically below 20 ppm

2.6 Sum Frequency generation spectroscopy (SFG)

SFG spectra were obtained by overlapping fixed visible (532 nm) and tunable IR (1000 – 4000 cm^{−1}) pulses, in time and space, using an EKSPLA Nd:YAG laser operating at 50 Hz that also pumped an EKSPLA optical parametric generation/amplification and difference frequency unit based on barium borate and AgGaS₂ crystals. Both beams had a bandwidth of 4 cm^{−1} and were focused to a diameter of ~2 mm at the sample surface at an energy of 160 μJ per pulse. All vibrational spectra were recorded in ppp (sum, visible, and infra-red) polarization combination and were collected at 4 cm^{−1} increments. Spectra can't be collected in the ssp mode from Au substrates because of mirror charge effects. The generated SFG signal was first filtered and dispersed by a monochromator and then detected with a gated photomultiplier tube. The resulting signal was normalized by both a reference signal generated in a ZnS crystal and, to eliminate water absorption artifacts, by a spectrum of a deuterated dodecanethiol self-assembled monolayer on gold. The fitting routine used for SFG data analysis has been described previously.^{41, 44}

2.7 Near-edge x-ray fine structure spectroscopy (NEXAFS)

NEXAFS N K-edge spectra were collected at the U7A beamline at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory, Upton, NY) using an elliptically p-polarized (~85%) beam. The beam line is equipped with a monochromator and a 600 l/mm grating that provides a full-width at half maximum resolution of ~0.15 eV at the carbon K-edge (285 eV). The monochromator energy scale was calibrated using the 398 eV N 1s p* transition on a titanium nitride grid,⁴⁵ while partial electron yields were divided by the beam flux of a gold grid during data acquisition.⁴⁶ A detector with a bias voltage of −360 V monitored partial electron yields. All samples were mounted to allow rotation about the vertical axis, thus, allowing the NEXAFS angle, the angle between the incident X-ray beam and the sample surface, to change. Previously, we ruled out the possibility of beam damage by repeatedly scanning between the 388 eV to 410 eV energy range while monitoring the partial electron yield at 398 eV.⁴¹ No changes in the spectra were observed within 15 minutes, which exceeded the exposure time in the longest nitrogen scan.

3. Results

In this study a small barrel (3 nm in height) shaped Protein G B1 variant, D4', was immobilized onto two different functionalized alkanethiols self-assembled onto gold substrates. Unlike the previous Protein G model built by Baugh *pet al.*,⁴⁰ the orientation of the D4' variant was controlled by electrostatic forces between the surface and the protein. Substitution of uncharged amino acids (asparagine and glutamine) for charged residues (aspartic acid and glutamic acid) was used to control the distribution of charge throughout the protein. For the D4' mutant used here, negatively charged amino acids at the N-terminus were replaced by mutations with neutral amino acids, creating a dipole like distribution with a negatively charged region near the C-terminus (Figure 1). Hence, immobilization of this protein onto positively (NH₂-SAMs) and negatively charged (COOH-SAMs) substrates should result in opposite end-on orientations. Since the protein adsorption is controlled by the electrostatic interactions nonspecific adsorption should be minimized.

To examine the degree of ordering as this protein is immobilized onto a charged surface, we collected SFG and NEXAFS data of a monolayer of D4' on a positively charged surface (NH₂-SAMs). SFG amide I spectra of a monolayer of D4' Protein G B1 on NH₂-SAM in air can be found in Figure 2. Within this amide stretching region are three features: an amide I vibrational mode near 1653 cm⁻¹ that is characteristic of ordered α -helices, and peaks near 1630 and 1680 cm⁻¹ that are characteristic of ordered β -sheet structures (B2 mode and B1/inter-strand mode).³¹ These features are absent from data collected from a disordered monolayer of wild-type protein.⁴⁰ As Baugh *pet al.* mentioned previously, because of the SFG selection rules and the fact that the amide bonds are symmetrically distributed in these anti-parallel sheets, it is surprising to observe the β -sheet stretching modes.⁴⁰ The phase of these peaks can also provide some qualitative insight into the orientation of the α -helix. All three of the features are in phase (positive peaks) with the non-resonant gold background. Previous SFG studies of helical peptides on alkanethiol SAMs found that when the peptide backbones were oriented parallel to the surface the spectral features corresponding to ordered α -helices are out of phase with the nonresonant gold background.³⁵ As a result, it can be assumed the helix within the D4' mutant is oriented upright on the surface.

The NEXAFS data collected provides an examination of the degree of orientation among secondary structural elements within the protein. N K-edge spectra of the protein films were acquired at three x-ray incidence angles between 20° and 70° (Figure 3). All spectra contain an adsorption edge, related to the excitation of N_{1s} electrons into continuum states, along with superimposed characteristic peaks. Each spectrum is dominated by a broad σ^* resonance near 405.5 eV, attributed to N-C bonds, and an intense transition at 401 eV assigned to amide groups.^{34, 47-49} The low-intensity pre-edge feature at 399 eV is characteristic of positively charged amine groups.^{41, 48, 50} Closer examination of the difference between the 20° and 70° spectra (Figure 3), reveals a negative dichroism for the resonance at 401 eV - indicative of some degree of protein ordering. Most prominent is the dichroism relating to the N_{1s} transition to the π^* orbital of the peptide bond (401 eV). While this π^* feature can originate from both α -helical and β -sheet structural units, the α -helix structures are not expected to contribute significantly to the angle dependence of amide peaks due to the broad distribution of orientations within the helix.³⁵ Hence, the peptide bonds in the antiparallel β -sheets should account for the majority of the observed negative dichroism. From the angle dependence of the amide π^* (using three spectra between 70° and 20°), average tilt angles of the amide π^* orbital relative to the substrate normal were calculated.⁴⁶ Such analysis produced an amide π^* orbital tilt angle of $42 \pm 5^\circ$. Assuming that the proteins form a perfectly aligned monolayer and that the amide π^* orbitals were oriented perpendicular to the N-C-O plane of the peptide bond, the β -strands have a uniform

distribution tilted 48° from normal. This corresponds well with the SFG data that shows the Protein G D4' is immobilized in an upright position on the amine surface.

As in the previous Protein G based model system with cysteine mutants, differences in orientation of the D4' mutant were probed using ToF-SIMS by monitoring the intensity of specific secondary ions stemming from amino acid residues at either end of the protein.⁴⁰ The secondary ion fragments examined includes methionine ($C_2H_5S^+$, $C_4H_{10}SN^+$) and tyrosine ($C_7H_7O^+$, $C_8H_{10}NO^+$) at the N-terminal region and Leucine/Isoleucine ($C_5H_{12}N^+$) at the opposite end (Figure 4, Table 1). However, it is important to note that the positive ion spectra from the bare NH_2 - and $COOH$ -SAMs has a significant $C_2H_5S^+$ peak originating from the thiol group at the tail of the molecule (Figure 1). To differentiate the contribution of the substrate-derived SAM $C_2H_5S^+$ and $C_4H_{10}SN^+$ peaks from the protein-derived methionine peaks, ^{13}C -labeled methionine was incorporated into D4' variant to create ^{13}C labeled peaks that were shifted by one mass unit. This labeling shifts the methionine $C_2H_5S^+$ and $C_4H_{10}SN^+$ peaks to $m/z = 62.0112$ and 105.0713 , respectively.

Again, Baugh *pet al.* showed that despite the complexity of ToF-SIMS data, the data can be reduced by just examining the ratio of the sum of intensities of N-terminal secondary ions, stemming from methionine and tyrosine, divided by the intensity of secondary ions stemming from leucine/isoleucine (Table 1).⁴⁰ To minimize concentration based matrix effects it is best to compare secondary-ion ratios between samples with similar protein coverages.³⁷ The protein surface concentration can be tracked by the XPS determined At % nitrogen. In this case, both the normalized D4'- NH_2 -SAM sample and the D4prime;- $COOH$ -SAM sample exhibit similar amounts of nitrogen, 5.6 ± 0.5 At % versus 6.4 ± 1.8 At % (Table 2), which corresponds to a monolayer of adsorbed protein.^{10, 17}

With the D4' protein immobilized on the NH_2 -SAMs the N-terminal region is expected to be pointed towards the vacuum interface, leading to an enrichment of secondary ions from the methionine and tyrosine residues. On the $COOH$ -SAMs, the C-terminal region is expected to be pointed towards the vacuum interface, leading to an enrichment of secondary ions from the leucine residues. Figure 5 shows calculated intensity ratios for the two different samples, D4' on NH_2 -SAMs versus D4' on $COOH$ -SAMs. Figure 5 illustrates a two-fold increase in this ratio (student's t-test: $p < 3 \times 10^{-10}$) between the charged Protein G B1 mutant on the two different SAM surfaces, indicating large changes in the intensities of secondary-ions originating from opposite ends on the protein. Just as in the previous investigation, the differences in this ratio can be interpreted as two opposite end-on protein orientations. Yet, this difference in calculated intensity ratios between the two different samples is two times larger than Baugh *pet al.* reported for the covalently-immobilized model system. This implies that that the electrostatic interactions do a better job of inducing this protein into two opposite orientations. While this may seem surprising, the covalent attachment system was just based on a single cysteine-maleimide bond, which could allow the protein sufficient mobility to produce a small range of orientations on the maleimide surface.

4. Conclusions

In this investigation, complementary ToF-SIMS, NEXAFS and SFG experiments provided detailed information about the orientation, chemical state, and molecular structure of a small model protein (charged Protein G B1 mutant) electrostatically immobilized onto two different SAM surfaces. SFG results and the angle dependence of amide features in the N K-edge NEXAFS spectra provided evidence of ordering of secondary structures within the protein. Differences in the intensities of secondary ions in the ToF-SIMS data demonstrated that the specific amino acid substitutions induced the protein into two different orientations. All of the data presented here were collected under vacuum or in air. As a result there could

be some denaturation of the protein, but the fact that changes in orientation are detected by ToF-SIMS and features related to ordered α -helices are detected by SFG implies that any denaturation is minimal.

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Figure 1. Schematic diagram of the Protein G1 B1 D4' variant immobilized via electrostatic interactions onto positively (NH₂-SAMs) and negatively (COOH-SAMs) charged substrates.

Figure 2.

SFG amide I spectra of a monolayer of D4' Protein G B1 on $\text{NH}_2\text{-SAM}$ in air. The amide I peak near 1653 cm^{-1} is characteristic of ordered α -helices while those near 1630 and 1680 cm^{-1} are characteristic of ordered β -sheet structures. These features are absent from data taken from a monolayer of wild-type protein on the same substrate (data not shown).

Figure 3.

NEXAFS spectra of the nitrogen K-edge for D4' Protein G B1 immobilized onto NH₂-SAMs, acquired at angles of 20° and 70°, along with the difference between 70° and 20° spectra. The prominent negative dichroism observed at 401 eV in the difference spectra is characteristic of amide bonds and is attributed to anisotropic β -sheet peptide bonds in Protein G B1.

Figure 4. Amino acids with asymmetric distributions used in ToF-SIMS analysis (shown in black). Isoleucine and leucine produce an identical characteristic positive ion fragment. The N-terminus and C-terminus are labeled N and C, respectively.

Figure 5.

ToF-SIMS peak intensity ratios for D4' Protein G B1 immobilized onto NH₂-SAMs and COOH-SAMs. Peak ratios were calculated as the sum of intensities of secondary ions from methionine (62 and 105 m/z) and tyrosine (107 and 136 m/z) divided by the sum of intensities of secondary ions from the leucine/isoleucine residues (86 m/z). Error bars represent the standard deviation across fifteen analysis spots over three distinct samples.

Table 1

Secondary ion fragments used in ToF-SIMS analysis

Source	Formula	Mass
Methionine	$^{13}\text{CCH}_5\text{S}^+$	62.0112
	$^{13}\text{CC}_3\text{H}_{10}\text{SN}^+$	105.0713
Tyrosine	$\text{C}_7\text{H}_7\text{O}^+$	107.0516
	$\text{C}_8\text{H}_{10}\text{NO}^+$	136.0836
Leucine/Isoleucine	$\text{C}_5\text{H}_{12}\text{N}^+$	86.1035

Table 2

Summary of XPS determined elemental composition of NH₂-SAMs, COOH-SAMs, and protein-coated substrates (atomic %). Average experimental compositions from three distinct samples (three spots per sample) are shown. All data were collected at a 0 TOA in the hybrid mode. Standard deviations are shown in parentheses

Sample	Atomic%					Normalized N
	Au	C	O	S	N	
Bare NH ₂ -SAMs	36.9 (1.5)	53.6 (1.8)	4.6 (0.8)	1.6 (0.3)	3.3 (0.5)	-
Bare COOH-SAMs	43.7 (2.5)	45.9 (2.4)	9.0 (0.3)	1.8 (0.2)	-	-
D4' on NH ₂ -SAMs	22.0 (1.0)	58.2 (1.4)	11.0 (0.3)	1.2 (0.2)	7.6 (0.5)	5.6 (0.5)
D4' on COOH-SAMs	27.3 (6.9)	53.4 (4.0)	11.5 (1.4)	1.4 (0.2)	6.4 (1.8)	-