

# Investigations of Lysozyme Adsorption at the Air/Water and Quartz/Water Interfaces by Vibrational Sum Frequency Spectroscopy

Gibum Kim, Marc Gurau, Joonyeong Kim, and Paul S. Cremer\*

Department of Chemistry, Texas A&M University, P.O. Box 30012,  
College Station, Texas 77843-3012

Received August 20, 2001. In Final Form: December 28, 2001

We used vibrational sum frequency spectroscopy (VSFS) to investigate lysozyme adsorption at the quartz/water and air/water interfaces. These investigations found that water structure depended intimately on bulk pH at both the hydrophobic, air, and hydrophilic, quartz, interfaces. Changes in the interfacial potential caused by protonation/deprotonation of titratable groups on the protein (as well as on quartz in the case of the solid/liquid studies) played a major role in this. Furthermore, intensity in the CH stretch range from lysozyme was observed under all conditions investigated at the air/water interface. Because only moieties that are well-aligned at the interface can be observed in VSFS, this was taken to be an indication that at least some lysozyme residues must be organized at the hydrophobic air surface. By contrast, CH stretches were not observed from protein adsorbed at the hydrophilic quartz/water interface unless the pH was raised above 8.0, causing the interface to take on a net negative charge. Even under very basic conditions, however, the CH stretch modes were much weaker in this case than those observed at the hydrophobic air/water interface.

## Introduction

Adsorption of proteins at interfaces has been a topic of increasing interest over the past several decades<sup>1–3</sup> because of its importance in fields ranging from medical implantation<sup>4</sup> and drug delivery<sup>5,6</sup> to biological sensor fabrication.<sup>7,8</sup> A variety of techniques have been employed to investigate adsorption behavior both at the solid/liquid<sup>9–15</sup> and vapor/liquid<sup>13,16–19</sup> interfaces. One protein

that has received particular attention is hen egg white lysozyme because the structural characteristics of this macromolecule are well-understood at the molecular level.<sup>20,21</sup> Lysozyme is composed of 129 amino acids, including 18 cationic and 12 anionic residues.<sup>22</sup> Lysozyme is considered to be a hard protein because four internal disulfide bonds help maintain its tertiary structure.<sup>23</sup>

The attention afforded lysozyme has already allowed many of its adsorption properties to be elucidated. For example, it is known that maximum protein adsorption at the air/water interface occurs near pH 11.0, the isoelectric point of this protein.<sup>19</sup> Furthermore, several studies<sup>23–25</sup> showed changes in surface tension as a function of time after the adsorption of lysozyme. These data were interpreted as the unfolding or partial denaturation of the protein caused by contact with the hydrophobic air surface. Other studies showed that a decrease in enzymatic activity was concomitant with this process.<sup>26</sup> It has also been revealed by neutron reflectivity and ellipsometry that the global conformation of the macromolecule can change from a “side-on” to a “head-on” orientation with respect to the molecule’s long axis as the interfacial density of the protein is increased.<sup>19,27</sup> The neutron reflectivity data also found no evidence for gross denaturation of the protein based upon layer thickness measurements.<sup>19</sup>

\* To whom correspondence should be addressed. Tel.: 979-862-1200. Fax: 979-845-7561. E-mail: cremer@mail.chem.tamu.edu.

(1) *Proteins at Interfaces: Physicochemical and Biochemical Studies*; Brash, J. L., Horbett, T. A., Eds.; ACS Symposium Series 343; American Chemical Society: Washington, DC, 1987.

(2) *Proteins at Interfaces II: Fundamentals and Applications*; Horbett, T. A., Brash, J. L., Eds.; ACS Symposium Series 602; American Chemical Society: Washington, DC, 1995.

(3) Tertykh, V. A.; Yanishpolskii, V. V. In *Adsorption on Silica Surfaces*; Papirer, E., Ed.; Surfactant Science Series; Marcel Dekker: New York, 2000; Vol. 90, p 523.

(4) Kenausis, G. L.; Voros, J.; Elbert, D. L.; Huang, N.; Hofer, R.; Ruiz-Taylor, L.; Textor, M.; Hubbell, J. A.; Spencer, N. D. *J. Phys. Chem. B* **2000**, 3298.

(5) Bae, Y. H.; Okano, T.; Kim, S. W. *J. Polym. Sci., Polym. Phys.* **1990**, 28, 923.

(6) Malmsten, M. *Colloids Surf., A* **1999**, 159, 77.

(7) Jones, V. W.; Kenseth, J. R.; Porter, M. D.; Mosher, C. L.; Henderson, E. *Anal. Chem.* **1998**, 70, 1233.

(8) Seigel, R. R.; Harder, P.; Dahint, R.; Grunze, M. *Anal. Chem.* **1997**, 69, 3321.

(9) Norde, W.; Favier, J. P. *Colloids Surf.* **1992**, 64, 87.

(10) Wahlgren, M.; Arnebrant, T.; Lundstrom, I. *J. Colloid Interface Sci.* **1995**, 175, 506.

(11) Robenson, J. L.; Tilton, R. D. *Langmuir* **1996**, 12, 6104.

(12) Norde, W.; Rouwendal, E. *J. Colloid Interface Sci.* **1990**, 139, 169.

(13) Ball, A.; Jones, R. A. L. *Langmuir* **1995**, 11, 3542.

(14) Su, T. J.; Lu, J. R.; Thomas, R. K.; Cui, Z. F.; Penfold, J. *J. Phys. Chem. B* **1998**, 102, 8100.

(15) Su, T. J.; Lu, J. R.; Thomas, R. K.; Cui, Z. F.; Penfold, J. *Langmuir* **1998**, 14, 438.

(16) Razumovsky, L.; Damodaran, S. *Langmuir* **1999**, 15, 1392.

(17) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, 70, 427.

(18) Postel, C.; Abillon, O. *Langmuir* **1998**, 14, 5649.

(19) Lu, J. R.; Su, T. J.; Howlin, B. J. *J. Phys. Chem. B* **1999**, 103, 5903.

(20) Blake, C. C. F.; Koenig, D. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, V. R. *Nature* **1965**, 206, 757.

(21) Shirahama, H.; Lyklema, J.; Norde, W. *J. Colloid Interface Sci.* **1990**, 139, 177.

(22) Clenfield, R. E.; Liu, A. K. *J. Biol. Chem.* **1965**, 240, 2000.

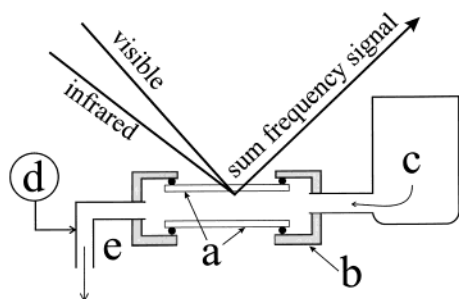
(23) Tripp, B.; Magda, J. J.; Andrade, J. D. *J. Colloid Interface Sci.* **1995**, 173, 16.

(24) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, 70, 403.

(25) Xu, S.; Damaodaran, S. *J. Colloid Interface Sci.* **1993**, 159, 124.

(26) Hunter, J. R.; Kilpatrick, P. K.; Carbonell, R. G. *J. Colloid Interface Sci.* **1990**, 137.

(27) Horsley, D.; Herron, J.; Hlady, V.; Andrade, J. D. In *Proteins at Interfaces: Physicochemical and Biochemical Studies*; Brash, J. L., Horbett, T. A., Eds.; ACS Symposium Series 343; American Chemical Society, Washington, DC, 1987; Chapter 19.



**Figure 1.** Schematic side-cut view of the flow cell in the VSFS experimental setup: (a) IR-grade fused quartz window; (b) Teflon flow cell body; (c) protein solution reservoir; (d) pH meter; and (e) drain from flow cell. The flow cell was replaced with a Langmuir trough for air/water interface studies.

In addition to air/water interface investigations, lysozyme adsorption studies have been carried out at the liquid/solid interface on fused quartz substrates. It was shown that adsorption also increased substantially in this case as the pH of the bulk solution was tuned through the isoelectric point.<sup>15,28</sup> Total internal reflection fluorescence microscopy<sup>11</sup> and attenuated total internal reflection FTIR studies<sup>13</sup> showed that conformational changes occurred on quartz substrates even below the isoelectric point of the protein. Furthermore, the amount of helical content of the protein decreased.<sup>29</sup> Finally, reorientation from "side-on" to "head-on" adsorption was observed in neutron reflectivity studies depending on the specific surface density.<sup>15</sup>

In this paper we have employed vibrational sum frequency spectroscopy (VSFS), a surface-specific nonlinear optical technique, to investigate lysozyme adsorption at both the quartz/water and air/water interfaces. VSFS is a particularly valuable technique for such studies because it is capable of monitoring the reorganization of interfacial water molecules upon the surface recruitment of a variety of adsorbates such as proteins,<sup>30</sup> polyelectrolytes,<sup>31</sup> and lipid bilayers<sup>32</sup> even in the presence of an overwhelming contribution of bulk water. Our investigations revealed that water structure was particularly sensitive to pH changes at both the hydrophobic, air, and hydrophilic, quartz, interfaces. Furthermore, intensity in the CH stretch range from lysozyme itself was observed under all air/water interface conditions investigated. This was a clear indication that hydrophobic lysozyme residues become aligned at this hydrophobic interface. More surprisingly, weak CH stretch intensity could also be observed from protein adsorption at the quartz/water interface, but only under high pH conditions where the overall surface charge was negative.

## Experimental Section

**Instrumentation.** VSFS experiments were performed with a passive-active mode-locked Nd:YAG laser (PY61c, Continuum, Santa Clara, CA) equipped with a negative feedback loop in the oscillator cavity to provide enhanced shot-to-shot stability. The 1064-nm beam had a pulse width of 21 ps and operated at a repetition rate of 20 Hz. It was used to pump an optical parametric generator/oscillator (OPG/OPA) stage (Laser Vision, Bellevue, WA) that generated the 532 nm and tunable infrared input beams (2800 to 4000  $\text{cm}^{-1}$ ) used in these experiments. Figure 1 shows the setup of the VSFS beams and sample stage. The IR and the visible beams were concentrically overlapped at the sample

interface with incident angles of 51° and 42°, respectively, with respect to the surface normal. All sum frequency spectra presented in this article have been taken with the SSP polarization combination, referring to the sum frequency, visible, and infrared beams, respectively. Each data set was normalized to spectra taken from a piece of Y-cut crystalline quartz.

Both the experimental and theoretical details of vibrational sum frequency spectroscopy have been described elsewhere.<sup>33–35</sup> Briefly, a sum frequency response is obtained, in the dipole approximation, only in media that lack inversion symmetry. In systems where the bulk is isotropic, the overwhelming majority of the signal is obtained at interfacial boundaries where inversion symmetry is necessarily broken. Vibrational spectra are obtained in VSFS experiments through resonance enhancements that occur as the infrared beam is tuned over vibrational modes of the surface species. As shown in eq 1, the sum frequency signal,  $I_{\text{SFS}}$ , is proportional to the square of the second-order nonlinear susceptibility,  $\chi^{(2)}$ .  $\chi^{(2)}$  can be broken into two parts, a frequency-dependent resonant term,  $\chi_{\text{R}}^{(2)}$ , and nonresonant term,  $\chi_{\text{NR}}^{(2)}$ ,

$$I_{\text{SFS}} \propto |\chi^{(2)}|^2 I_{\text{vis}} I_{\text{IR}} = |\chi_{\text{R}}^{(2)} + \chi_{\text{NR}}^{(2)}|^2 I_{\text{vis}} I_{\text{IR}} \quad (1)$$

where  $I_{\text{vis}}$  and  $I_{\text{IR}}$  denote the visible and IR beam intensities, respectively. The resonant term can be expressed as follows,

$$\chi_{\text{R}_n}^{(2)} = \sum_n \frac{A_n}{\omega_{\text{IR}} - \omega_n + i\Gamma_n} \quad (2)$$

where  $A_n$ ,  $\omega_n$ ,  $\omega_{\text{IR}}$ , and  $\Gamma_n$  are the oscillator strength, resonant frequency, frequency of the IR beam, and damping constant of the  $n$ th vibrational resonant mode, respectively.  $A_n$  is the product of the infrared and Raman transition dipole moments as well as a phase factor, their orientational vector average, and their number density at the interface. This is essentially the heart of the sum frequency selection rules giving rise to interfacial sensitivity. Namely, the vast majority of the signal is derived from an ordering of dipoles, which occurs readily at interfaces.

**Materials.** Purification of lysozyme was accomplished by size exclusion chromatography with a Sephadex G-50 column. After sample preparation, gel electrophoresis showed the presence of only 1 band at about 11 400 Da corresponding to the molecular weight of lysozyme. Polyglycine (molecular weight 4600) and polyalanine (molecular weight 1850) were used as received. The protein, polypeptides, and column were all purchased from Sigma. The concentrations of lysozyme solutions were determined by absorption measurements at 280 nm in a UV/visible spectrometer. The water used in preparing all buffers and cleaning solutions for these experiments was obtained from a NANOpure Ultrapure Water System (Barnstead, Dubuque, IA) and had a minimum resistivity of 18  $\text{M}\Omega\cdot\text{cm}$ . The  $\text{D}_2\text{O}$  (ISOTEC Inc., Miamisburg, OH) used was 99.9% isotopically pure. Buffers at the desired pH values were made by dissolving appropriate amounts of sodium phosphate into solution. The final pH was adjusted to within 0.1 pH units of the desired value by adding HCl or NaOH. Sufficient NaCl was added to all buffers to raise the total ionic strength to 0.030 M. Because the concentration of lysozyme added to the buffers was always 1 mg/mL, it added <0.001 M to this value.

A Langmuir trough (Model 601M, Nima Technology Ltd., Coventry, England) equipped with a Wilhelmy plate was used for the air/water experiments. Several hours were allowed for each lysozyme solution to equilibrate. Equilibrium was judged to have been approached when no further changes could be detected by either surface pressure or VSFS measurements. Liquid/solid interface adsorption experiments were performed in a home-built flow cell with a total volume of about 2 mL. The flow cell consisted of a machined Teflon body containing two open faces on the top and bottom to which infrared-grade fused quartz windows (Quartz Plus Inc., Brookline, NH) were fixed. Before attachment, the windows were cleaned in hot chromic acid solution for several hours, rinsed with copious amounts of water, and baked overnight in a kiln at 500 °C. Experiments

(28) Norde, W. *Adv. Colloid Interface Sci.* **1986**, 25, 267.

(29) Norde, W.; Giacomelli, C. E. *Macromol. Symp.* **1999**, 145, 125.

(30) Kim, J.; Cremer, P. S. *ChemPhysChem* **2001**, 8/9, 543.

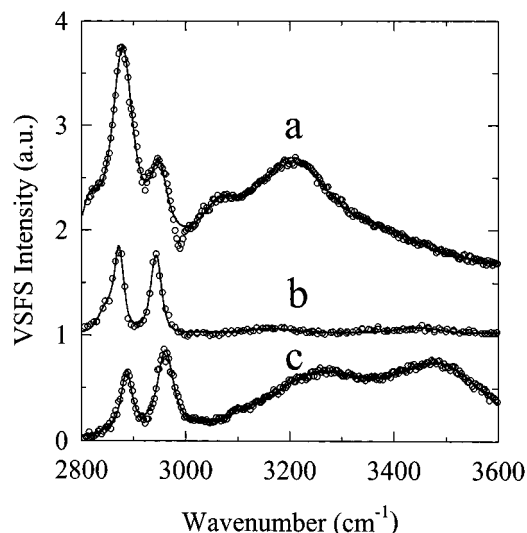
(31) Kim, J.; Cremer, P. S. *J. Am. Chem. Soc.* **2000**, 122, 12371.

(32) Kim, J.; Kim, G.; Cremer, P. S. *Langmuir* **2001**, 17, 7255.

(33) Miranda, P. B.; Shen, Y. R. *J. Phys. Chem. B* **1999**, 103, 3292.

(34) Richmond, G. L. *Anal. Chem.* **1997**, 17, 5536A.

(35) Bain, C. D. *J. Chem. Soc., Faraday Trans.* **1995**, 91, 1281.



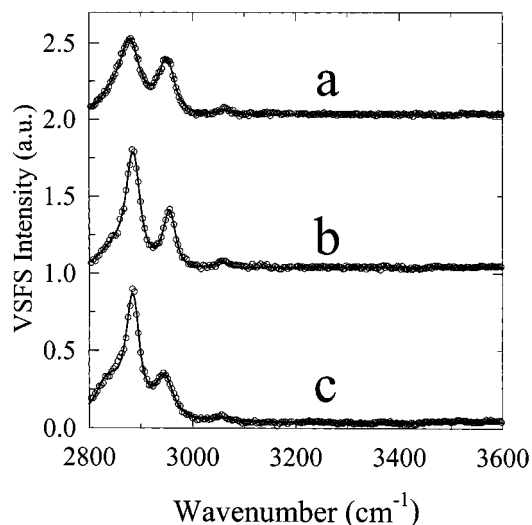
**Figure 2.** Sum frequency spectra in the CH and OH stretch range at the air/water interface upon lysozyme adsorption at pH (a) 3.2, (b) 11.0, and (c) 12.3. Spectra (a) and (b) were offset from (c) by 2 and 1 units of intensity, respectively.

were carried out by first introducing protein-free buffer solution into the cell followed by a lysozyme solution at the same pH and ionic strength. In this case, it was only necessary to wait  $\approx 30$  min before the VSFS spectrum stopped changing. At this point excess lysozyme was carefully rinsed out with the original buffer. Sum frequency spectra were obtained both before and after rinsing away excess lysozyme. Very little difference was noted and, therefore, only spectra after rinsing out are presented here.

**Data Acquisition.** A data acquisition program written in Labview 5.0 (National Instruments, Austin, TX) was used to acquire data sets for each experiment. The fitting of each VSFS spectrum was performed with a Voigt profile by a program written in MatLab version 5.3 with the optimization toolbox version 2.0 (Mathworks Inc, Natick, MA).

## Results

**Air/Water Interface.** Sum frequency spectra of lysozyme were obtained at the air/water interface as shown in Figure 2 at pH (a) 3.2, (b) 11.0, and (c) 12.3. The solid lines represent least-squares fits to the raw data using a Voigt function. In Figure 2a the broad feature around  $3200\text{ cm}^{-1}$  is associated with the OH stretch of interfacial water tetrahedrally coordinated to neighboring molecules and is often referred to as the “ice-like peak”.<sup>36</sup> There was little evidence in these spectra for the presence of substantial amounts of more weakly hydrogen-bonded water, which often gives rise to a feature near  $3450\text{ cm}^{-1}$  and is referred to as the “water-like peak”.<sup>36</sup> Also, no evidence was found for a free OH stretch at  $3690\text{ cm}^{-1}$ . The overwhelming intensity of the ice-like feature in comparison with the water-like feature is not surprising as lysozyme is highly positively charged under these conditions with a net charge of +11 per protein.<sup>37</sup> Therefore, when protein molecules adsorb at the interface, they create a substantial electric field that strongly orients interfacial water. On the other hand, at pH 11.0 (Figure 2b), which is very near the isoelectric point of lysozyme, neither the ice-like nor water-like peaks give rise to much intensity, even though maximum protein adsorption is expected<sup>19</sup> because the interfacial charge has been largely eliminated. Furthermore, above the isoelectric point at pH 12.3 (Figure 2c), the water-like peak becomes prominent as the net field is much weaker than that at pH 3.2.



**Figure 3.** Sum frequency spectra in the CH and OH stretch range at the air/D<sub>2</sub>O interface upon lysozyme adsorption at pH (a) 3.2, (b) 11.0, and (c) 12.3. Spectra (a) and (b) were offset from (c) by 2 and 1 units of intensity, respectively.

In this case there is a net charge of  $-2$  per protein,<sup>37</sup> although somewhat more protein is adsorbed than that at pH 3.2.<sup>19</sup>

Because the charge on the protein switches from positive to negative as the pH is raised from 3.2 to 12.3, it is expected that the net orientation of the water dipoles should reverse as well. A strong piece of evidence for this claim can be seen in the nature of the interference between the OH stretch peaks and those in the CH stretch region below  $3000\text{ cm}^{-1}$ . Namely, there is a dip near  $3000\text{ cm}^{-1}$  due to destructive interference between the OH and CH stretch peaks in Figure 2a,<sup>38</sup> while there is substantial intensity in this region in Figure 2c. Furthermore, the CH stretch features in Figure 2c appear a bit blue-shifted with respect to the other two spectra. These results are analogous to previous data by Gragson and co-workers in which they showed very similar interference behavior in the presence of positively and negatively charged surfactant monolayers at the air/water interface.<sup>39–41</sup>

The presence of the two large features between  $2850$  and  $3000\text{ cm}^{-1}$  in the CH stretch region in Figure 2 is almost certainly due to the alignment of hydrophobic moieties on the amino acid residues at this hydrophobic vapor/liquid interface. This might be expected because adsorption at the air/water interface may include partial unfolding or conformational changes. Indeed, hydrophobic parts of lysozyme have been thought to reorient themselves to maximize their interactions with the hydrophobic medium.<sup>42,43</sup> Because these CH stretch peaks clearly have a substantial amount of interference with the neighboring OH stretch features, the adsorption experiments were repeated in the corresponding D<sub>2</sub>O buffers to obtain more accurate information on their intensities and positions (Figure 3). All three spectra in Figure 3 give evidence for

(38) The Voigt fit to the spectrum in Figure 2a does not take into account the phase difference between the CH and OH stretches and therefore does not properly fit the dip.

(39) Gragson, D. E.; Richmond, G. L. *J. Am. Chem. Soc.* **1998**, *120*, 366.

(40) Gragson, D. E.; McCarty, B. M.; Richmond, G. L. *J. Am. Chem. Soc.* **1997**, *119*, 6144.

(41) Gragson, D. E.; McCarty, B. M.; Richmond, G. L. *J. Phys. Chem.* **1996**, *100*, 14272.

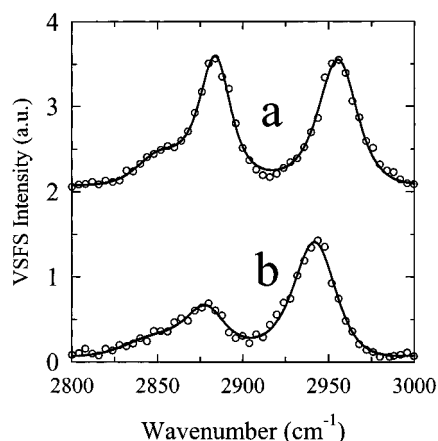
(42) Douillard, R. *Thin Solid Films* **1997**, *292*, 169.

(43) Holt, S. A.; McGillivray, D. J.; Poon, S.; White, J. W. *J. Phys. Chem. B* **2000**, *104*, 7431.

(36) Du, Q.; Freysz, E.; Shen, Y. R. *Phys. Rev. Lett.* **1994**, *72*, 238.

(37) Tanford, C.; Roxy, R. *Biochemistry* **1972**, *11*, 2192.



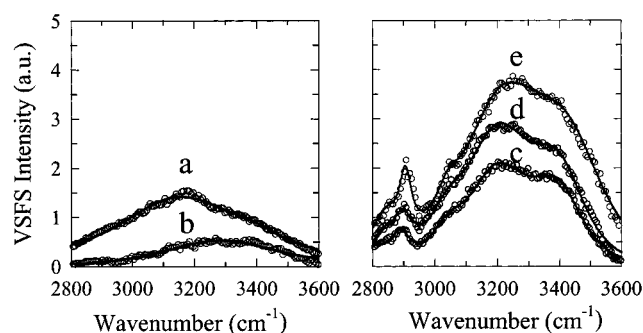


**Figure 4.** Sum frequency spectra in the CH and OH stretch range of (a) polyalanine and (b) polyglycine at the air/water interface. Spectrum (a) was offset from (b) by 2 units of intensity.

two prominent features near 2887 and 2952  $\text{cm}^{-1}$ . To help with the assignment of these features, CH stretch range reference spectra were taken of polyglycine and polyalanine spread at the air/water interface (Figure 4). Polyglycine, which contains a methylene but not a methyl moiety, gives rise to features near 2884 and 2955  $\text{cm}^{-1}$ . Polyalanine, which contains a methyl group and a methine (C–H) group, but no methylene units, give rise to features near 2878 and 2942  $\text{cm}^{-1}$ . We therefore conclude that the two major peaks seen in Figure 3 are likely due to a mixture of hydrocarbon moieties. The very weak feature near 3062  $\text{cm}^{-1}$  may be from an aromatic CH stretch mode by analogy with previous sum frequency spectra of monolayers containing aromatic functional groups;<sup>44,45</sup> however, additional reference spectra of peptides such as polyphenylalanine failed to show sufficient intensity at this frequency to make any definitive assignment. Nonetheless, it is this weak intensity that is probably giving rise to a small 3050  $\text{cm}^{-1}$  feature seen in the  $\text{H}_2\text{O}$  experiment in Figure 2a and an even weaker and somewhat blue-shifted ( $\approx 3100 \text{ cm}^{-1}$ ) feature in Figure 2c. In addition to the three CH stretch peaks discussed above, fits of the data to a Voigt function provide evidence for an additional low-frequency shoulder near 2847  $\text{cm}^{-1}$ .

A comparison of the intensities from the spectra in Figure 3 reveals that the CH stretches are weakest at pH 3.2 (Figure 3a). This is likely due to the fact that less material is adsorbed under these very acidic conditions than at higher pH.<sup>19</sup> Fitting the data from Figure 3b,c indicates that the features at pH 12.3 are slightly stronger than those at pH 11.0. This is curious because more protein is adsorbed at the isoelectric point. It is known from neutron reflectivity experiments<sup>19</sup> that some conformational change takes place as the pH is raised above 11.0 and this may be giving rise to the slightly better alignment observed in our VSFS experiments. Indeed, the increasing intensity ratio of the 2878  $\text{cm}^{-1}$  peak relative to the 2942  $\text{cm}^{-1}$  peak as the pH is raised in Figure 4 seems to support the idea that some reorientation takes place.

**Quartz/Water Interface.** In a second set of experiments lysozyme was adsorbed at the quartz/water interface to test the effect of hydrophilic conditions on the adsorption process. Lysozyme solutions were flowed into the cell and excess protein was rinsed out with pure buffer at the same pH value. Figure 5 shows sum frequency



**Figure 5.** Sum frequency spectra of lysozyme adsorbed at the quartz/water interface at pH (a) 3.2, (b) 8.0, (c) 9.0, (d) 10.0, and (e) 11.0.

spectra of lysozyme adsorbed at pH (a) 3.2, (b) 8.0, (c) 9.0, (d) 10.0, and (e) 11.0. The data were split into two sections only for the sake of clarity. As was the case at the air/water interface, intensity can again be seen in the OH stretch region near 3200 and 3450  $\text{cm}^{-1}$ . The OH features were most intense under the most basic conditions (Figure 5e). As the pH was lowered, these peaks reached a minimum value near pH 8.0 (Figure 5b) and then begin to rise again under more acidic conditions (Figure 5a).

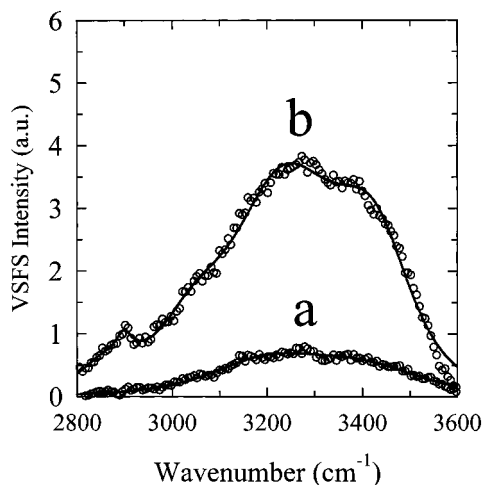
The origins of the OH stretch features are more complicated at the quartz/water interface than at the air/water interface because lysozyme and the underlying quartz substrate both contribute to the interfacial potential. The surface charge on the quartz emanates from the deprotonation of surface silanol groups and has already been studied in great detail.<sup>36</sup> These previous investigations have shown that, in the absence of surface adsorbed species, the OH stretch peaks increase steadily as the pH is raised from 3.0 to 12.0. This is because progressively more surface silanol moieties become deprotonated and thus the interfacial charge density increases. In the presence of lysozyme, however, the OH stretch features are of moderate intensity at low pH, decrease to a minimum near pH 8.0, and then rise again above this value. This behavior is due to the fact that both lysozyme and surface silanols are affecting the interfacial potential. Indeed, the net charge on lysozyme is known to be about +14 at pH 3, +7 at pH 8, and –2 at pH 12.<sup>37</sup> On the basis of the OH stretch intensities in Figure 5, the lysozyme/quartz system is almost certainly closest to its overall isoelectric point near pH 8.0, where negative charge from the quartz and positive charge from the lysozyme effectively cancel. Above pH 8.0 the interface is negatively charged and below this value it is positively charged. Previous studies of the  $\xi$ -potential during lysozyme adsorption further support this argument.<sup>12,21,46</sup>

Interestingly, some weak signal near 2900  $\text{cm}^{-1}$  can be seen from the quartz/water interface above the isoelectric point in Figure 5c–e. In fact, the intensity increases steadily as the pH is raised from 9.0 to 11.0, although the nature of the interference between the CH and OH stretches in VSFS makes it difficult to quantify this. The presence of these peaks seems to indicate that individual residues in the protein layer become oriented as the negative charge on the quartz increases. Such observations can be made with nonlinear optical experiments such as VSFS that *only* give rise to a signal from an alignment of dipoles at the interface. When species are very poorly aligned or are isotropic, no signal is obtained. This is the case when the net charge at the interface is positive and

(44) Bell, G. R.; Li, Z. X.; Bain, C. D.; Fischer, P.; Duffy, D. C. *J. Phys. Chem. B* **1998**, *102*, 9461.

(45) Watry, M. R.; Richmond, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 875.

(46) Haynes, C. A.; Sliwinsky, E.; Norde, W. *J. Colloid Interface Sci.* **1994**, *164*, 394.



**Figure 6.** Sum frequency spectra of the quartz/water interface after initially adsorbing the protein at pH 3.2 and raising the pH to (a) 8.0 and (b) 11.0.

therefore dominated by the charge on the lysozyme and as a result no CH stretch range signal is observed. Apparently, a sufficient negative charge on the underlying quartz substrate such that it dominates the net sign of the surface potential is able to impose some limited alignment on the lysozyme. The amount of signal in the CH stretch range seen at this hydrophilic interface is, however, very small compared with the data from the air/water interface (Figures 2 and 3). Indeed, control experiments performed at pH 11 in D<sub>2</sub>O buffer solutions (data not shown) gave rise to a CH stretch signal that was more than an order of magnitude weaker than corresponding data for the air/D<sub>2</sub>O interface shown in Figure 3.

Despite the weak intensity of the CH features in Figure 5, protein alignment can be imposed by raising the pH above 8.0 from any initial starting condition. Figure 6 shows an example of this behavior. Lysozyme was originally adsorbed at pH 3.2 and the pH was subsequently raised to (a) 8.0 and (b) 11.0. As can be clearly seen, CH stretch intensity is present under the more basic conditions. The reason the 2900 cm<sup>-1</sup> signal is weaker than that observed upon direct adsorption at pH 11.0 is probably because substantially less protein adsorbs at the quartz/water interface at pH 3.2 than at 11.0.<sup>15</sup>

### Discussion

VSFS studies of lysozyme at the air/water interface indicated that some alignment of protein residues occurs over the entire pH range investigated as evidenced by the presence of CH stretch intensity from aligned CH<sub>x</sub> moieties. Indeed, methyl, methylene, and methine moieties probably align normal to the interface to maximize their contact with air and minimize their contact with water. This is consistent with previous dynamic surface tension measurements,<sup>23</sup> which indicated a decrease in surface tension takes place upon lysozyme adsorption. Neutron reflectivity studies,<sup>19</sup> however, indicated that gross protein structural changes do not take place in this macromolecule at the air/water interface under similar conditions. It therefore appears likely that the CH stretch

intensity represents local alignment of CH<sub>x</sub> moieties that are present on the local portion of the protein that is exposed to air rather than rearrangements involving internal amino acid residues. This is consistent with the rigid nature of this protein. The change in the intensity ratio of the 2878 and 2952 cm<sup>-1</sup> peaks seen in Figure 4 as the pH is raised from 3.2 through 11.0 to 12.3 is an indication that the nature and/or orientation of these residues is changing. Indeed, neutron reflectivity data indicated that the protein changes its orientation from side-on to head-on as the pH is raised to 11.0 because of increasing surface concentration.<sup>19</sup> It is not clear if the sensitivity of the interfacial water structure to changes in pH also plays a role in this.

The results of lysozyme adsorption at the quartz/water interface show evidence for structural rearrangements of the protein above pH 8.0 (Figure 5), where the entire interface takes on a net negative charge. The vibrational spectra in the CH stretch range in this case show evidence for a dominant CH stretch range feature near 2900 cm<sup>-1</sup>, while all spectra at the air/water interface show two distinct features that are well-shifted from this frequency (Figures 2 and 3). This should not be completely surprising as the mechanism for CH<sub>x</sub> alignment at the charged solid interface may be entirely different. Indeed, it is expected that the large electric field emanating from deprotonated silanol groups on the substrate surface should be largely responsible for reorienting charged residues within the protein. Very different residues may therefore be aligned in this process than the ones that are oriented at the hydrophobic air/water interface.

### Conclusions

We have shown evidence for water structural rearrangements at both the quartz/water and air/water interfaces upon lysozyme adsorption. At the air/water interface, these changes are dominated by the net charge on the lysozyme at any given pH. The net dipole moment of the water at the interface flips directions if lysozyme is adsorbed at a pH value above rather than below its isoelectric point. Evidence for this comes from the constructive and destructive interference seen between the OH and CH stretches around 3000 cm<sup>-1</sup>. At the quartz/water interface, the combined charges on the lysozyme and quartz determine the nature of the water structure with a minimum OH stretch signal seen near pH 8.0. CH stretch intensity is seen at all pH values at the air/water interface, where hydrophobic residues on the lysozyme surface probably reorient. By contrast, no CH intensity is seen at the quartz/water interface below pH 8.0. Above pH 8.0, the lysozyme/quartz system takes on a net negative charge and CH stretch intensity is observed, although it is far weaker than that at the air/water interface.

**Acknowledgment.** This research was generously supported by the Robert A. Welch Foundation (Grant A-1421), the National Science Foundation (CHE-0094332), the Petroleum Research Fund (Grant 34149-G5), a Research Innovation Award from the Research Corporation of America (Grant RI0437), and an ONR-YIP Award (N00014-00-1-0664).

LA0113365