The behavior of individual molecules of R-phycoerythrin (RPE) was monitored by fluorescence imaging at various pHs and ionic strengths within the evanescent-field layer (EFL) at a water/fused-silica interface. Above the isoelectric point (pI), the individual protein molecules moved between exposures with random motion. As the pH approached the pI of the protein, the RPE molecules were partially adsorbed onto the fused-silica surface. The residence time and the number of molecules within the EFL also increased near the pI. Below the pI, the protein molecules were completely and permanently adsorbed onto the surface. However, the observed number of distinct molecule spots was decreased somewhat because of aggregation. At a given buffer condition, plots of residence times and molecule numbers exhibit asymmetry nearly identical to the corresponding elution peaks of the proteins in capillary electrophoresis and capillary liquid chromatography. These results provide insights into the fundamental interactions for the adsorption/desorption of proteins at the liquid/solid interface.

Direct observation of single molecules can provide insight into molecular genetics,1–3 biochip assembly,4–7 biosensor design,8–10 DNA biophysics,11–15 and basic separation mechanisms of capillary electrophoresis (CE) and liquid chromatography (LC).26–31 While it is well known that both electrostatic and hydrophobic interactions govern protein adsorption at liquid/solid interfaces,32–36 relatively little is known about how these interactions influence the individual protein molecules in the interfacial layer.26 These fundamental properties are the foundation for many chromatographic20,33,34 and electrophoretic protein separation theories.26,33,34 If both electrostatic and hydrophobic interactions govern protein adsorption at liquid/solid interfaces, which interaction is stronger? What external factors influence the behavior of individual protein molecules on the surface? These questions can be answered by the imaging of single-protein molecules to follow their real-time dynamics.26,30 Some previous work reported imaging of single biomolecules labeled with fluorescent dye23 or via native fluorescence.33,35–37 However, adsorption and desorption events were not studied in those experiments. Single-molecule imaging has been employed to study static adsorption coverage on a chromatographic surface.39 Adsorption/desorption dynamics have been

recorded using photon bursts coupled with epifluorescence excitation. Motion at a water/fused-silica interface has been imaged to reveal electrostatic trapping of molecules. As a model protein, we selected R-phycocerythrin (RPE), which is a 240-kDa autofluorescent protein consisting of 7 subunits and ~30 chromophores. Phycobiliproteins such as RPE, B-phycocerythrin (BPE), and allophycocyanin (APC) are stable and highly water-soluble globular proteins derived from cyanobacteria and eukaryotic algae. They are slightly acidic with isoelectric points around pH 5 and are stable over the pH range 5–9. The pI of RPE depends on the source of the protein. The pI of RPE from Rhodella violacea was reported to be between 4.2 and 4.4, D’Agno and others reported two pairs of major bands at pI 4.30–4.35 and at pI 4.50–4.55, and minor component at pI 5.70–5.75 in RPE from G. longa. Orta-Ramirez et al. reported the pI of RPE from Porphyra yezoensis as 4.1, 4.2, and 6.1, respectively. Although the pI is a very important property of a protein, the exact pI values of RPE derived from cyanobacteria and eukaryotic algae have yet to be reported.

In this study, we describe the real-time imaging of individual protein molecules of RPE at a water/fused-silica interface without fluorescence dye labeling. Single proteins were imaged by a total internal reflection fluorescence microscopy technique. The distribution and motion of individual protein molecules at the interface were analyzed as a function of pH and buffer composition. The results of the imaging experiments were also compared to the elution behaviors of proteins in CE and LC. Finally, the correlation between the pI obtained from isoelectric focusing experiments and single-protein dynamics is discussed. Unlike the previous study, external fluorescence labels were avoided through the use of a natively fluorescent protein. This way, contributions from sample heterogeneity due to multiple labeling and adsorption of the fluorescence label are eliminated entirely.

**Experimental Section**

**Buffer Solutions.** The various buffer systems used are as follows: sodium acetate/acidic acid (pH 4.6), Gly-His/NaOH (pH 7.0), and Gly-Gly/NaOH (pH 8.2). Gly-Gly, Gly-His, and sodium hydroxide were ACS grade purchased from Sigma Chemical Co. (St. Louis, MO). Sodium acetate buffer solutions were prepared at various pHs using 1 M solutions of acetic acid, sodium acetate, and sodium chloride. ACS grade or higher glacial acetic acid, sodium acetate, and sodium chloride (all from Fisher Scientific, Fairlawn, NJ) were dissolved in ultrapure 18-MΩ water using the same procedure as described by Kang et al. In each case, the final mass balance of acetate is 25 mM as is the nominal ionic strength. All other chemicals were ACS grade. All solutions were filtered through a 0.2-µm filter prior to use.

![Figure 1. Schematic diagram of the experimental setup for monitoring single-protein molecules within the evanescent field layer. Laser, argon ion laser; AO, acoustooptic modulator; PH1, pinhole selection of first-order diffracted beam; PR, equilateral prism to remove plasma lines; M1–3, steering mirrors; PH2, 3, laser alignment pinholes; L, plano-convex lens; and MI, microscope.](image)

Protein Sample Preparation. RPE is a stable and water-soluble protein derived from cyanobacteria and eukaryotic algae and was obtained from Molecular Probes (Eugene, OR). All RPE samples were prepared at a concentration of 16.7 nM in 10 mM Gly-Gly buffer without fluorescence dye labeling. For the single-molecule imaging experiments, these RPE samples were further diluted to 16.7 pM immediately prior to the start of the experiment. For CE and capillary liquid chromatography (CLC), RPE samples were diluted to 333.4 pM immediately prior to injection into the capillary.

Determination of Isoelectric Point. For isoelectric focusing (IEF), the RPE sample was centrifuged at 5000 rpm for 10 min, after which the pellet was dissolved in 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5, and then dialyzed with a 66383 Pierce Slide-A-Lyzer Dialysis Cassette (Rockford, IL) in a cold chamber (5°C) overnight. For isoelectric focusing, the dialyzed RPE samples were directly loaded onto a PhastGel IEF 4–6.5 separation medium (Pharmacia Biotech, Uppsala, Sweden), which contained polyacrylamide bonded to a transparent polyester backing. The isoelectric point (pI) of RPE was determined by isoelectric focusing in a PhastSystem (Pharmacia) operated at 2 kV and 15°C. The protein was detected by coomassie staining as described in the PhastSystem Development Technique File No. 200. The pI was determined by comparing the relative mobility of protein bands to that of a protein standard IEF mix (pH 3.6–6.6, Sigma) run on the same gel.

Evanescent Wave Excitation Geometry. The excitation geometry was similar to that previously reported (Figure 1). The sample chamber for single-protein molecule experiments was...
constructed by sandwiching a 4-μL volume of sample between a No. 1 (22 mm square) Corning glass cover slip and the hypotenuse face of a right-angle fused-silica prism (Melles Griot, Irvine, CA; prism UVGFS, A = B = C = 25.4 mm). A focused laser beam was directed through the prism toward the prism/sample interface. The incident angle θı was directed through the prism toward the prism/sample interface. The incident angle θı was greater than the critical angle θc defined by Snell’s law. In this system, θı was slightly greater than 66°.

**Microscope and ICCD Camera.** A Pentamax 512-EFT/1EIA intensified CCD (ICCD, Roper Scientific, Princeton, NJ) camera was mounted on top of a Zeiss Axioskop upright microscope. The digitization rate of the camera was 5 MHz (12 bits) with software controller gain set at 3 and hardware intensifier gain set at 10. The camera was operated in the external synchronous mode with the intensifier disabled open. The frame-transfer mode was employed. The excitation source was a Coherent Innova-90 argon ion laser operated at 488 nm. Laser light was passed through an isomol model 1205C-2 acoustooptic modulator that was optimized for maximum output in the first order according to the manufacturer instructions. Exterraneous light and plasma lines from the laser were eliminated prior to its entry into the observation region with the aid of an equilateral prism and an optical pinhole. The light was then focused with a 30-cm-focal length plano-convex lens such that the focal point was at the fused-silica/water interface described above. For imaging, a Plan-Neofluar objective lens (100×, 1.3 oil NA, Zeiss) was used. The objective was optically coupled to the cover slip with immersion oil (type FF, Cargille, Cedar Grove, NJ). Two 488-nm holographic notch filters (Kaiser Optical, HNFP) with optical density of >6 were used between the objective and the ICCD. Timing was controlled with a frame rate of 10 Hz using a Stanford Research Systems model DG535 four-channel digital delay/pulse generator. The ICCD camera was triggered at time 0 with a 10-ms duration TTL pulse.

**CE and CLC.** CE and CLC analyses were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA). A fused-silica capillary (Polymer Micro Technologies, Inc., Phoenix, AZ) of 75μm i.d., 375μm o.d. with 48-cm effective length and 58-cm total length was used. The running buffers were the same set of solutions used for the single-protein molecule imaging experiments. In CE, the RPE sample was introduced with low pressure (0.5 psi = 3.4 × 10^4 Pa) for 3 s at the anodic end of the capillary. The applied electric field was +344.8 V/cm at 25 °C. After each run, the capillary was rinsed in the following sequence: water; 0.1 M NaOH; water; and running buffer for 4 min at 20 psi. In CLC, the RPE sample was injected with low pressure for 3 s and eluted with low pressure at 25 °C without applying an electric field. In both CE and CLC, RPE fluorescence (LIF) was excited with a Beckman Coulter 488-nm Laser M odule and detected at an emission wavelength of 580 nm. P/ACE MDQ Software (version 2.3) was used for system control, data collection, and data processing. All peaks were recorded in the direct fluorescence detection mode.

**RESULTS AND DISCUSSION**

**Determination of pI.** In isoelectric focusing of our protein sample, three distinct bands appeared with measured pIs of 4.3, 4.4, and ~4.7, respectively (Figure 2). The presence of similar but distinct bands suggested that several isomers could coexist in the protein sample. These pIs were slightly different when compared to the pIs of purified phycobiliproteins such as RPE, BPE, and APC (all around pH 5). Although our protein samples were all RPE, the pIs did not correlate exactly with those reported by others. This confirmed that the pI of RPE depends on the source of the protein. As RPE is the major phycobiliprotein in most red algae phycobilisomes and has three dissimilar subunits, the α, β, and γ polypeptides, these results suggest that the RPE protein derived from cyanobacteria and eukaryotic algae also contained these various subunits or isomers.

**Single-Protein Molecules at a Fused-Silica Surface.** Series of native fluorescence images of RPE molecules at the water/fused-silica interface were recorded in the pH range of 4.0–8.2 (Figure 3A). For pH ≥ 7.0, all of the molecules were randomly moving between exposures. Molecules appeared and disappeared at different locations on successive images due to movement in and out of the EFL. The signal-to-noise ratios (S/N) of the molecule spots were low because on the average RPE only spent a fraction of a millisecond within the evanescent-field layer (EFL). Molecules also moved within the image over short distances due to Brownian motion, producing globular spots that reflect the integrated trajectory over the exposure time. This shows that the individual protein molecules were located inside the EFL rather than immobilized at the solid surface. At pH ~6.0, most of the RPE molecules were still fluctuating in solution. However, a few molecules (~2%) started to adsorb onto the fused-silica prism surface (Figure 3B). These show up as brighter spots (long residence time) and appear at the same image location over more than one frame. This phenomenon showed that at least one isomer (presumably associated with the pI ~4.7 minor band in Figure 2) of RPE had a weakened electrostatic repulsive force at pH 6.0 and can adsorb onto the surface. At pH 5.5, most of the protein molecules were adsorbed

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and the fused-silica surface at this pH led to the adsorption of the reduction of electrostatic repulsion between the protein molecules protein spots never moved back into the solution. Again, the are lower than the pH (Figure 3C). The individual disk-shaped fused-silica surface at pH 5.0 even though the p
photobleached.
The single-molecule spots showed a higher S/N because fluo-
rescence was collected for longer times and over fewer pixels due to adsorption. This also confirms that the molecules in Figure 3A and B were moving in and out of the EFL rather than being photobleached.

All of the protein molecules were strongly adsorbed on the fused-silica surface at pH 5.0 even though the pI of RPE isomers are lower than the pH (Figure 3C). The individual disk-shaped protein spots never moved back into the solution. Again, the reduction of electrostatic repulsion between the protein molecules and the fused-silica surface at this pH led to the adsorption of the molecules onto the prism surface. Below pH 4.1 (< pl), the charge of all of the RPE subunits (or isomers) were positive since the pIs of RPE were 4.3, 4.4, and ~4.7, respectively (Figure 2). But, the surface silanol groups of fused silica remained in the dissociated form SiO− at pH 4.0. Electrostatic attraction between the oppositely charged surface and RPE dominated the interaction. Thus, at pH 4.0, the protein molecules were completely and permanently adsorbed onto the prism surface (Figure 3D). Because the electrostatic interaction between protein molecules and the prism surface was much stronger than the repulsive force between the individual protein molecules, the protein molecules formed aggregates. The RPE molecules were still globularly shaped but appeared as much larger spots (Figure 3D). The number of protein molecules within each aggregate is not uniform. For the largest group in Figure 3D, the integrated intensity is about 10–15 times that of the smaller (presumably single-molecule) spots. It appears that once proteins adsorb onto the surface, more protein molecules tend to adsorb at the same location. Thus, a quadratic concentration dependence of the spot size is not observed. One explanation is that the interaction among the (roughly neutral) protein molecules is stronger than that between proteins and the fused-silica surface.

The total number of protein molecules recorded over several frames versus pH for 16.6 pM RPE in the pH range of 4.0–8.2 was determined (Figure 4). Generally, for pH < pl of the protein, a substantial increase in the number of molecules was observed at the water/fused-silica interface. However, the total number of RPE molecules recorded at pH 4 (< pl of RPE) was smaller compared to those for pH 5 and 5.5. The reason that the maximum number of molecules adsorbed was at pH ~5.0 rather than at pH ~4.0 is as follows: above the pl, the negatively charged protein molecules were moving between exposures with random motion. As the pH approached the pl, the residence times and the number of the molecules within the EFL increased due to decreased electrostatic repulsion. The molecules can adsorb onto the prism surface near the pl even when the pH was higher than the pl of protein. Below the pl, the protein molecules were completely and permanently adsorbed onto the fused-silica surface because of strong electrostatic attraction. However, the observed number of molecule spots decreased because of the aggregation of molecules. From the estimated aggregation number above, the left entry in Figure 4 is consistent with the general trend for the other pH conditions. The individual spot sizes (pixels) increased toward lower pH (Figures 5 and 6). Although some molecules at pH 5 were still distributed in the range of 4–20 pixels (white bars in Figure 5), its average spot size per molecule increased as pH decreased (Figure 6). Larger spot size indicates longer trapping time within the EFL. This provides confirmation that the interaction of a protein molecule at the liquid/solid interface at the single-molecule level fits the long-range trapping theory rather than permanent adsorption except at the lowest pH.

**Comparison with Chromatography.** Since the trapping of single molecules within the EFL could explain chromatographic retention and electrophoretic migration of proteins, DNA, or both on a fused-silica surface, the elution peak shapes of RPE in

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**Figure 3.** Images of native fluorescence from RPE molecules in (A) 10 mM Gly-Gly (pH 8.2), (B) 25 mM sodium acetate/acetic acid (pH 6.0), (C) 25 mM sodium acetate/acetic acid (pH 5.0), and (D) 25 mM sodium acetate/acetic acid (pH 4.0) buffer solutions. Images were acquired with 9.9-mW laser power and 10-Hz exposure. The concentration of RPE was 16.7 pM.

**Figure 4.** Histogram of single-protein molecules recorded versus pH for 16.7 pM RPE in pH 4.0–8.2 buffer solutions. Each data point represents the mean value of number of molecules observed from three independent measurements. ICCD exposure time was 10 ms at 10 Hz.
CLC and CE were studied in the range of pH from 4.0 to 8.2. In CLC, RPE showed different peak shapes and retention times depending on the applied pressure in a 75-μm-i.d. capillary with 10 mM Gly-Gly buffer (pH 8.2) (Figure 7). Peak broadening in a fused-silica capillary in CLC indicated the adsorption of protein molecules. Both the asymmetry ratio (α) of the peaks and the retention times of RPE decreased with increasing pressure (Table 1). This implied that the adsorption of protein molecules to the fused-silica capillary inner wall increased as the pressure-driven flow decreased, as predicted by the rate of mass transfer to the stationary phase. In a series of CLC runs at 1.0 psi pressure-driven flow above pH 4.0, the pH did not affect the retention time but caused a slight increase in asymmetry at and below pH 5.5 (Figure 8 and Table 2). The peak area gradually decreased below pH 5.0. The peak did not elute at all at pH 4.0. These results show a one-to-one correspondence between LC of proteins and the single-protein adsorption behavior at a water/fused-silica interface.

Comparison with CE. In CE, the retention times and the peak shapes of RPE were severely affected by the pH in contrast to the CLC experiments. This is because CE peaks are inherently much sharper and subtle changes can be readily discerned. The protein peaks became broader with decreasing pH, and the

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**Table 1. Asymmetry Ratios and Retention Times of RPE Peaks Eluted from a Fused-Silica Capillary at Different Pressures in CLC**

<table>
<thead>
<tr>
<th>applied pressure (psi)</th>
<th>asymmetry ratio (α)</th>
<th>retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 (= 3.4 × 10³ Pa)</td>
<td>3.27</td>
<td>7.02</td>
</tr>
<tr>
<td>0.8 (= 5.4 × 10³ Pa)</td>
<td>1.25</td>
<td>4.30</td>
</tr>
<tr>
<td>1.0 (= 6.8 × 10³ Pa)</td>
<td>1.07</td>
<td>3.39</td>
</tr>
</tbody>
</table>

* CLC conditions: hydrodynamic injection for 3 s at 0.5 psi (= 3.4 × 10³ Pa); bare fused-silica capillary 58 cm × 75 μm i.d. (48 cm to the detector); running buffer, 10 mM Gly-Gly buffer (pH 8.2); sample concentration, 333.4 pM RPE.  
  b Asymmetry ratio (α): ratio of the peak width at half-height (W½) before and after the peak maximum.

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**Table 2. Asymmetry Ratios and Retention Times of RPE Peaks Eluted from a Fused-Silica Capillary at Various pH in CLC**

<table>
<thead>
<tr>
<th>pH</th>
<th>asymmetry ratio (α)</th>
<th>retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>nonelution</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>1.46</td>
<td>3.50</td>
</tr>
<tr>
<td>5.0</td>
<td>1.35</td>
<td>3.52</td>
</tr>
<tr>
<td>5.5</td>
<td>1.30</td>
<td>3.53</td>
</tr>
<tr>
<td>6.0</td>
<td>1.07</td>
<td>3.52</td>
</tr>
<tr>
<td>7.0</td>
<td>1.03</td>
<td>3.47</td>
</tr>
<tr>
<td>8.2</td>
<td>1.07</td>
<td>3.39</td>
</tr>
</tbody>
</table>

* CLC conditions: hydrodynamic injection for 3 s at 0.5 psi (= 3.4 × 10³ Pa); bare fused-silica capillary 58 cm × 75 μm i.d. (48 cm to the detector); 1.0 psi of pressure-driven flow. Sample concentration, 333.4 pM RPE.  
  b Asymmetry ratio (α): ratio of the peak width at half-height (W½) before and after the peak maximum.
retention times increased dramatically (Figure 9). The triangular-shaped peaks with fronting at pH 8.2 and 7.0 are due to mismatch in electrophoretic mobilities compared to those of the buffer ions. Curiously, distortion of the RPE peak appeared at the front of the peak in the pH range 5.0–6.0 (Figure 9B–E). At pH 4.5, the peak shape of RPE showed severe peak tailing (Figure 9F), and finally, RPE did not elute at pH 4.0 (Figure 9G).

Traditionally, it has been assumed that the peak shape was due to either electromigration dispersion or the interaction of the analyte with the capillary inner wall. However, these peak shapes and asymmetry ratios (Table 3) were different from previously reported values for the real-time dynamics of single-DNA molecules undergoing adsorption and desorption at a liquid/solid interface or the asymmetry of RPE protein peaks in capillary zone electrophoresis. We suggest that this difference resulted from the composition of RPE and its peptides, and therefore, they have different net charges depending on the pH. At pH 6.0 and above, the net charge of all subunits (isomers) is negative. Between pH 5.0 and 5.5, even though RPE still has negative charge, three overlapping peaks are obtained for the subunits (isomers). The fronting asymmetry (Figure 9C–E) is due to the differential adsorption of RPE isomers on the fused-silica capillary inner wall. These results correlated exactly with the single-protein molecule experiments in 25 mM sodium acetate buffer pH 5.0–6.0 (Figure 3). At pH 4.5, although the isomers of the two RPE subunits (or isomers) were still lower than the pH of the buffer solution, the electrostatic repulsion between the RPE subunits and the fused-silica surface decreased. Therefore, RPE adsorbed onto the capillary wall and caused the tailing asymmetry (Figure 9F). The nonelution of RPE at pH 4.0 resulted from the complete and permanent adsorption of RPE molecules onto the capillary wall when the protein became fully positively charged. This last process was not observed previously even at the lowest pH studied.

### CONCLUSION

We demonstrated imaging of native single-protein molecules at a liquid/solid interface. The residence times and the number of the protein molecules within the EFL increased near the pl of the protein. Plots of residence times and total numbers of protein molecules exhibit nearly identical asymmetry as the corresponding elution peaks and track the nonelution behavior of the protein near the pl in CE and in CLC. Even though RPE has three subunits (α, β, γ) or isomers with different pl, the major factor for adsorption of RPE molecule at the water/fused-silica surface was the electrostatic interaction between the protein and the surface. These results are a direct verification of the statistical theory of CE and CLC at the single-protein molecule level with the caveat that long-range trapping is the dominant mechanism. This long-range interaction is confirmed by the adsorption of protein molecules at a fused-silica surface and by the generation of dual flow in an electrophoresis channel. Unlike our previous study, where concanavaline A never permanently adsorbed on fused silica and was always eluted in CE over the pH range studied, RPE does adsorb at the lowest pH here. The difference is likely due to the lower ionic strengths used, the presence of large numbers of fluorescence labels, or both in the previous experiments. It is also possible that the exposed hydrophobic surfaces of these proteins are very different in area and in distribution.

### ACKNOWLEDGMENT

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