Probing Adsorption, Orientation and Conformational Changes of Cytochrome *c* on Fused Silica Surfaces with the Soret Band†

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The Soret absorption band has been utilized as a probe for the adsorption of cytochrome *c* to the surface of a fused silica prism in direct contact with bulk protein solutions of various concentrations and pH. Employing linear polarized light and a single-pass total internal reflection absorption technique, we examined in detail the adsorption isotherm, molecular orientation, packing density, and conformational change of the protein bound to the bare (hydrophilic) and silanized (hydrophobic) glass surfaces. An adsorbate density of $\Gamma = 1.4 \times 10^{13}$ molecules/cm^2 was determined for the hydrophilic substrate at pH 7.2 and $C_b = 110$ μM, indicating that the protein molecules are essentially closely packed on the surface at saturation. The packing density is sensitive to the solution pH as well as the surface hydrophobicity, a result that the protein−surface interaction is governed by both electrostatic and hydrophobic forces. The same forces also govern the molecular orientation, yielding an angle of $\theta_\mu = 41°$ between the heme plane and the surface normal at neutral pH. The angle is retained over a wide pH range (4−9) and is fairly independent of the surface coverage on both the hydrophilic and hydrophobic substrates. Reorientation of the protein occurs ($41° \rightarrow 20°$) at pH $\approx$ 3, when the cyt *c* unfolds and the hydrophobic force becomes dominant in the adsorption process.

Introduction

Numerous biotechnologies involve proteins at interfaces. Examples include biocompatible materials, protein chromatography, solid-phase immunoassays, biosensors, and biochips.[1] Studying proteins at interfaces with spectroscopic means allows a detailed examination of the nature of protein−surface interactions as well as a stringent test of the principles that govern the conformational change and functionality of proteins within confined spaces.[2−5] Such knowledge is crucial to both technological and methodological developments in many biomedical applications.[6]

In studying proteins at interfaces, Fourier transform infrared (FTIR) spectroscopy in combination with total internal reflection (TIR) is a commonly used method.[7−10] The method is adopted to investigate the backbone amide groups, whose vibrations reflect the change in α-helix and β-sheet secondary structures of the adsorbed proteins. No information, however, is provided by such spectra about the molecule's adsorption orientation. Raman spectroscopy has also been applied to the investigation of proteins at interfaces. This technique is particularly sensitive for heme-containing proteins, which have a strong absorption arising from the electronic transition ($\pi \rightarrow \pi^*$) of the heme moiety, ideal for resonance-enhanced Raman excitation in the optical region.[11] In addition to the information about structural changes as provided by TIR−FTIR, molecular orientation of the adsorbed protein can be deduced from the relative intensity of the in-plane transitions of the heme group using light that is linearly polarized in two different orientations.[12]

For the heme proteins, Saavedra and co-workers[13] have studied extensively the molecular orientation of both cytochrome *c* (cyt *c*) and myoglobin (Mb) on the substrates of variable surface chemistry. They determined the orientation of the transition dipoles in the heme using linear dichroism for a protein film on the surface of an integrated optical waveguide and assessed the molecular orientation distribution from laser-induced fluorescence anisotropy measurements conducted in the TIR configuration. Their results indicated that the mean molecular orientation of both cyt *c* and Mb on both the hydrophilic and hydrophobic glass substrates is anisotropic rather than random. From the crystallographic dimensions of each protein molecule, monolayer densities of $\sim 1.3 \times 10^{13}$ and $\sim 0.9 \times 10^{13}$ molecules/cm^2 were estimated for cyt *c* and Mb, respectively, assuming that no "spreading" results from adsorption-induced conformational changes.

Salafsky and Eisenthal[14] recently showed that second harmonic generation (SHG) spectroscopy is a useful tool in probing protein adsorption to the fused silica surface and to negatively charged phospholipids bilayers. The SHG signal can reflect the amount of the surface-bound protein because binding of the protein molecules to the surfaces reduces the polarization of interfacial water, resulting in the loss of the SHG signal. The technique is intrinsically surface-selective and has allowed a detection sensitivity on the order of 10^{11} molecules/cm^2 to be achieved explicitly for cyt *c*. The result suggests that IR−vis sum frequency generation (SFG) spectroscopy[15] is also a feasible approach to the investigation of cyt *c* adsorbed on the surfaces.

An adsorbed protein molecule changes its conformation to some extent because of protein−surface interactions. Specifi-

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cally, a surface may lower the energy state of the partially "unfolded" protein relative to the energy of the corresponding conformations in solution.[16] However, this unfolding or spreading effect is often very small and difficult to probe. Even under the most extreme conditions, Herbold et al.[16] observed only a small percentage of cyt *c* unfolding on an anionic sulfopropyl support when examining the effect of temperature on the elution properties of the protein in hydrophobic interaction chromatography. Their findings suggested that the conformational changes similar to those observed in solution can occur at lower temperatures (by ~20 K) when cyt *c* binds to a surface.

We propose a new method to study the combined phenomena of adsorption isotherms, molecular orientation, packing density, conformational changes, and pH dependence of cyt *c* bound to the fused silica surfaces with high sensitivity. The method involves the use of total internal reflection absorption (TIRA) spectroscopy, focusing on the Soret band of the heme moiety.[11] The Soret band has an exceptionally large molar absorptivity[17] and has been known to be a sensitive probe for the protein's conformational changes (induced by acid, alcohol, and other denaturing reagents) in solution.[18−20] Particularly, the shift of this band reveals the conditions of ligand binding, oxidation states of the iron, and conformations of the polypeptide in the vicinity of the porphyrin group. Compared to TIRF (total internal reflection fluorescence) spectroscopy,[13,21−24] TIRA is advantageous in being able to provide precise measures of the absolute number of surface-bound proteins without the need for calibration against appropriate external standards.[23a] By using this method, information about the adsorbate density as well as the adsorption-induced conformational changes can be deduced from a close comparison of the protein's properties in solution and on the surface.

Experimental Section

Sample Preparation. Horse heart cyt *c* was obtained commercially (Sigma) and used without further purification. The protein was first dissolved in deionized water, purified with a Milli Q plus system (Millipore, resistivity > 18 MΩ/cm), to prepare a 1 mM stock solution. The solution was then diluted with 7 mM phosphate buffers (Acros) of various pH's to the desired acidity and concentration in each experiment. Concentrations of such prepared protein solutions were determined spectroscopically by referring to the molar absorptivity of the Soret band for the oxidized form of cyt *c*: $\epsilon_b = 1.06 \times 10^5$ M^{-1} cm^{-1} at $\lambda = 408$ nm and pH 7.[25] The acidity of the solutions was measured using a calibrated pH meter (Beckman Coulter Φ390).

Surface Preparation. The totally reflecting face of a right-angle fused silica prism (CVI) served as the glass surface. The surface has a specified flatness of $\lambda/10$ ($\lambda = 632$ nm) and was used as received. Prior to the measurement, the prism was thoroughly cleaned with standard cleaning solutions, rinsed extensively with deionized water, and dried with spectroscopic-grade acetone to produce a hydrophilic surface. The hydrophobic surface was prepared by liquid-phase silanization[13,26] after cleaning the bare glass surface with the mixed solution of H_2SO_4/H_2O_2 (2:1) for more than 30 min. Silanization was carried out by immersing the surface in a 2% solution of dimethyldichlorosilane (Fluka) in dry toluene (Aldrich) for 2 h. After the silanization, the surface was rinsed extensively with reagent-grade ethanol and deionized water. Both the hydrophilic and hydrophobic silica prisms were kept in a plastic bag purged with N_2 before use.

Spectroscopic Measurements. The Soret absorption spectra of free and surface-bound protein molecules were acquired with a UV−vis spectrophotometer (Hitachi U-3200). A quartz cell with a path length of 1 mm (Lightpath Optical) was employed for the bulk solution measurements, and a UV-graded fused silica prism (CVI) in a single-pass TIR arrangement[27−29] was used for the measurement of the surface-bound proteins. Figure 1a shows the experimental setup in which the prism is inserted into the compartment of the spectrophotometer in a manner similar to that of the solution sample cell. This allows the two setups to be interchanged readily. In the TIRA measurement, the evanescent wave detected the adsorbate via the internal reflection of the light through the right-angle prism on which a static sample cell was situated. The cell was made of a modified viton O-ring (~25 mm diameter), separating the prism from a glass plate. The space (3 mm) created by the O-ring can be filled with the desired solution in the individual measurements or emptied between the measurements using a small pipet without significantly altering the position of the sample holder. We started the measurements with sample solutions of the lowest pH or lowest concentration, and the spectra were acquired by scanning the spectrometer from 500 to 350 nm with a spectral bandwidth of 2 nm and a scan speed of 15 nm/min. Linear dichroism measurements were conducted using a dichroic polarizer (OptoSigma), which has a specified extinction ratio of 1×10^{-4} over the wavelength range of 380−700 nm. The polarizer was mounted on a rotary precision stage (Newport) for selection of the light polarization.

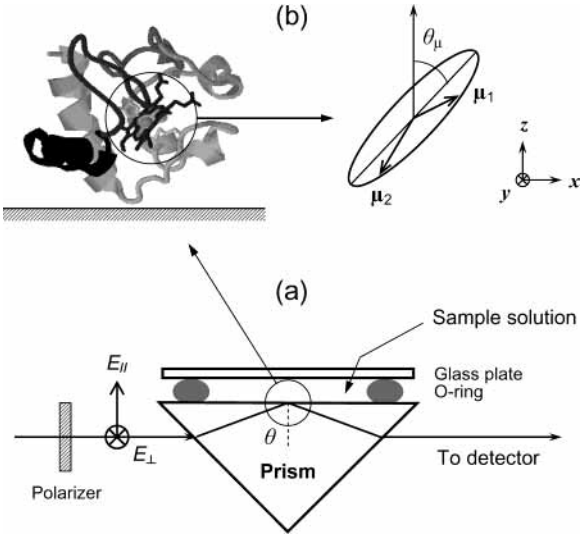

Figure 1. (a) Optical arrangement of the fused silica prism for single-pass attenuated total reflection absorption measurements. (b) Schematic of the plane of the porphyrin ring containing two degenerate electronic transition dipoles (μ_1 and μ_2) and the laboratory coordinate system defined by the *x*, *y*, and *z* axes. The structure of cyt *c* shown on the left was adapted from ref 48.

All of the absorption spectra were collected at room temperature. Because the Soret band absorbance detected in this experiment is typically very low, $A < 0.02$, special care has been taken to avoid unnecessary changes in the positions of the prism as well as the polarizer to maintain the consistency of optical alignment throughout the experiment. By doing so, a detection sensitivity better than $\Delta A \approx \pm 0.001$ can be routinely achieved. No measurement was made at pH > 8 for the hydrophobic surface because the silanized material is unstable in the alkaline solution.[30]

Results and Discussion

Adsorption Isotherm. Figure 2 shows the absorption spectra of cyt *c* on the hydrophilic surface in the wavelength range of

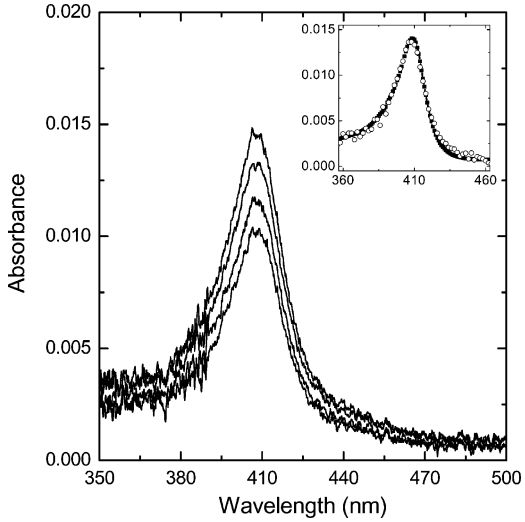

Figure 2. Optical absorption spectra of cyt *c* adsorbed to the hydrophilic fused silica surface at various bulk protein concentrations, $C_b = 4.0$, 5.8, and 30 to 110 μM (bottom to top) at pH 7.2 in 7 mM phosphate buffer. (Inset) Comparison of the Soret absorption bands of cyt *c* in solution (■) and on the surface (○) at $C_b = 75$ μM after proper scaling of the solution-phase spectrum. All of the spectra were acquired using randomly polarized light.

350−500 nm at bulk concentrations of $C_b = 4-110$ μM at pH 7.2 in 7 mM phosphate buffers. The Soret band is seen to peak at 408 nm with its shape staying nearly the same over the entire concentration range of investigation. The difference between this band and that of the protein in solution is negligible (inset in Figure 2), indicating that no profound conformational change occurs as the protein is adsorbed to the surface at neutral pH. A similar result was obtained for cyt *c* on the hydrophobic surface (data not shown).

To quantify the protein adsorption, the contribution of the free (or unbound) protein molecules to the observed band intensity should be assessed first. It is well established that the light beam used in the TIR mode has a finite penetration depth and that it probes both adsorbed and free protein molecules in the vicinity of a surface.[31] In this assessment, we follow Jang and Miller[32] for the analysis of the penetration depth (d_p) of the evanescent wave as

$$d_p = \frac{\lambda}{2\pi n_1(\sin^2\theta - n_{21}^2)^{1/2}} \tag{1}$$

with an effective path length (d_e) of

$$d_e = \frac{d_{e\perp} + d_{e\parallel}}{2} \tag{2}$$

which is an average of the sample thickness measured in the parallel (|| or TM) and perpendicular (⊥ or TE) polarizations

$$d_{e\parallel} = \frac{2n_{21}d_p\cos\theta(2\sin^2\theta - n_{21}^2)}{(1-n_{21}^2)[(1+n_{21}^2)\sin^2\theta - n_{21}^2)]} \tag{3}$$

$$d_{e\perp} = \frac{2n_{21}d_p\cos\theta}{1-n_{21}^2} \tag{4}$$

where λ is the wavelength of the light in a vacuum, n_{21} is the ratio of the refractive indices of the sample versus those of the TIR crystal, and θ is the angle of incidence. With the setup depicted in Figure 1a, we have $\theta = 73.8°$ at $\lambda = 408$ nm, $n_2 \approx 1.33$ for the solution, and $n_1 = 1.47$ for silica, which yield a penetration depth of $d_p = 139$ nm and an effective thickness of $d_e = 424$ nm when randomly polarized light is used in the measurement. Assuming a step profile for the bulk protein concentration[32] of

$$A_b = \epsilon_b C_b d_e \tag{5}$$

we have $A_b \approx 4.5 \times 10^{-4}$ with $\epsilon_b = 0.106$ μM^{-1} cm^{-1} at $C_b \approx 100$ μM. This absorbance is essentially within the limit of our experimental error (±0.001) and needs to be taken into account only when $C_b \geq 250$ μM or when the interaction between cyt *c* and the surface is repulsive, which occurs at the two extreme pH regions.

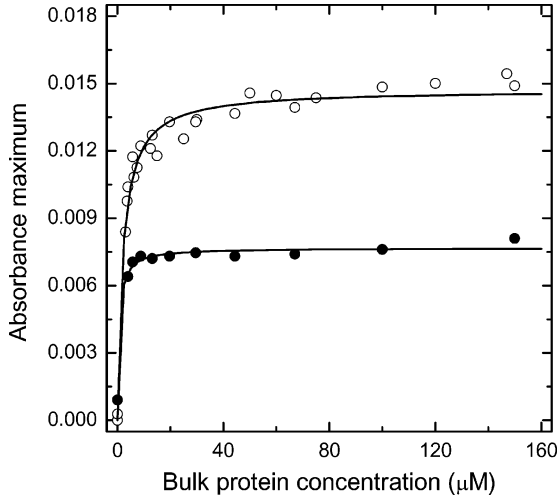

Figure 3. Adsorption isotherms of cyt *c* on the hydrophilic (○) and hydrophobic (●) fused silica surfaces at pH 7.2. The solid curve is the best fit of the experimental data to the Langmuir adsorption isotherm with $K_a = 0.5 \times 10^6$ and 1.5×10^6 M^{-1} (see eq 6 in text) for curves denoted by ○ and ●, respectively.

Figure 3 shows the adsorption isotherm plotted in terms of the Soret absorbance maximum (A_m) at $\lambda = 408$ nm versus C_b. The maximum is seen to increase steadily with the bulk protein concentration and gradually levels off as C_b approaches 70 μM. The data points can be well fit to the Langmuir adsorption model

$$\Theta = \frac{K_a C_b}{1 + K_a C_b} \tag{6}$$

where Θ is the ratio of the number of occupied adsorption sites to the total number of sites at saturation and K_a is the adsorption equilibrium constant. The same adsorption pattern can be found for cyt *c* on the hydrophobic surface, although the amount of protein adsorbed is significantly reduced at saturation (Figure 3). The observation of this saturation behavior clearly indicates that the detected signals are indeed derived from surface-bound proteins rather than from free protein molecules in solution.

Conformational Changes. Proven by circular dichroism, vibrational spectroscopy, and fluorescence spectroscopy, the conformation of cyt *c* in free solution may be altered by changing the acidity, temperature, ionic strength, and concentration of the denaturing reagent (e.g., acid, methanol, urea, etc.).[18−20] The surface itself may serve as a catalyst to change the protein's conformation. However, as pointed out earlier, the surface-induced effect is small and is practically undetectable at neutral pH for cyt *c*. To enhance this effect, denaturation of

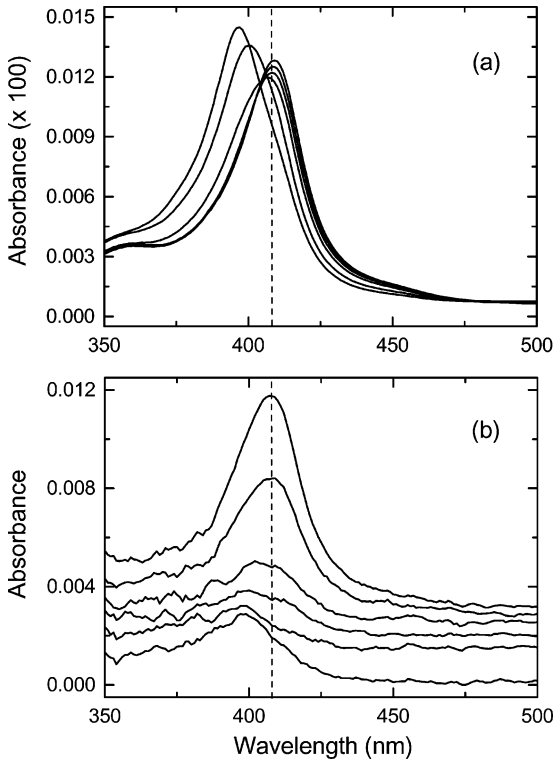

Figure 4. Optical absorption spectra of cyt *c* (a) in free solution and (b) on the hydrophilic surface at pH 2.4, 2.7, 2.9, 3.3, 3.7, and 4.7 (left to right in a and bottom to top in b). The protein concentration was fixed at 110 μM in 7 mM phosphate buffer. The spectra in b are shifted along the vertical axis for clarity, and the dashed lines denote the wavelength $\lambda = 408$ nm. All of the spectra were acquired using randomly polarized light.

the protein with acids was examined on both the hydrophilic and hydrophobic surfaces.

An examination of the surface-assisted acid-induced protein unfolding started with the bulk solution of various pH's. Figure 4a shows some sample spectra of cyt *c* in bulk protein solution, taken at a concentration of 110 μM using a 1-mm path length. The peak is observed to shift from 409 to 397 nm as the pH of the solution decreases from 10 to 2.5 (Figure 5). A sharp transition is found between pH 2.5 and 3.0, in accord with previous measurements.[18,19] The blue shift of the Soret band has been attributed to the unfolding of the protein, which leads to the displacement of the protein ligands from the heme iron and the replacement of the ligands by water molecules.[25]

The corresponding spectra of cyt *c* adsorbed on the hydrophilic glass surface are plotted in Figure 4b for comparison with the solution results. The Soret maximum decreases steadily with solution pH ranging from 9 to 2. As will be discussed in the next two sections, this is a result of the lessening of the electrostatic attraction between cyt *c* and the bare glass surface. Here we focus our attention on the pH-dependent band shift, particularly in the lower pH region, where differences are noticed between these free and surface-bound proteins. Although the Soret band stays at nearly the same position (408 nm) for the free protein molecules, the Soret band of the surface-bound proteins already shows a shift to the blue at pH 3.2.

To provide a more complete picture, we compare in Figure 5 the shifts of the Soret band of cyt *c* both on the hydrophilic surface and in the bulk solution over a wide pH range. These two sets of data are essentially the same from pH 5−10. Significant differences are found at pH < 4, where the folding/

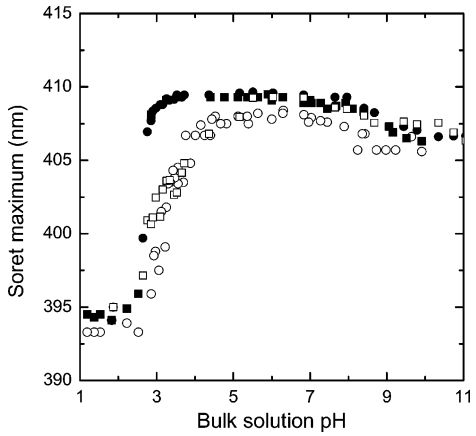

Figure 5. Shifts of the Soret absorption bands of cyt *c* in free solution (■ and ●) and on the bare glass surface (□ and ○) as a function of solution pH. Squares and circles represent two independent measurements.

unfolding transition point of the surface-bound protein is shifted to higher pH (i.e., from $pH_t \approx 2.7$ in solution[19b] to $pH_t \approx 3.2$ on the hydrophilic surface). An implication of this small but significant shift ($\Delta pH_t \approx 0.5$) is that the heme protein tends to be unfolded more readily upon adsorption to the hydrophilic glass surface. This observation is in accord with the finding of Goheen and co-workers,[16] who similarly detected the preferential unfolding of cyt *c* on an anionic surface only under extreme conditions by high-performance liquid chromatography.

Molecular Orientation. The amount of cyt *c* on the surface (i.e., the packing density) was determined directly from the observed spectra. This determination is justified because the investigation conducted in the earlier section revealed minimal conformational changes of the protein upon adsorption to the surfaces. However, to determine the packing density precisely, the molecular orientation must be known because the molar absorptivity of a surface-bound protein molecule, which is spatially oriented, may or may not be equal to its solution-phase value, depending on the polarization of the light used in the linear dichroism measurement.[12,33] Following Saavedra and co-workers,[13] we define the dichroic ratio (ρ) as

$$\rho = \frac{A_{f,||}}{A_{f,\perp}} = \frac{A_{t,||} - A_{b,||}}{A_{t,\perp} - A_{b,\perp}} \tag{7}$$

where $A_{t,||}$ ($A_{t,\perp}$) and $A_{f,||}$ ($A_{f,\perp}$) are the total absorbance and the absorbance of the protein film acquired at the parallel (perpendicular) polarization, respectively. For the Soret band with transition dipoles polarized in the plane of the porphyrin ring, the ratio is related to the orientation of the planar heme moiety by[22]

$$\rho = \frac{E_x^2}{E_y^2} + \frac{E_z^2}{E_y^2}\left(\frac{2\cos^2\theta_\mu}{1 + \sin^2\theta_\mu}\right) \tag{8}$$

where θ_μ is the angle of the porphyrin plane tilted away from the surface normal of the substrate (cf. Figure 1b) and E_x, E_y, and E_z are the respective electric fields of the evanescent wave along the *x*, *y*, and *z* axes:

$$E_x = \frac{2\cos\theta(\sin^2\theta - n_{21}^2)^{1/2}}{(1-n_{21}^2)^{1/2}[(1+n_{21}^2)\sin^2\theta - n_{21}^2)]^{1/2}} \quad (9)$$

$$E_y = \frac{2\cos\theta}{(1-n_{21}^2)^{1/2}} \quad (10)$$

$$E_z = \frac{2\cos\theta\sin\theta}{(1-n_{21}^2)^{1/2}[(1+n_{21}^2)\sin^2\theta - n_{21}^2)]^{1/2}} \quad (11)$$

Figure 6a shows the spectra acquired from the hydrophilic surface for two different polarizations at $C_b = 110\ \mu$M and pH 7.2 under comparison. As shown, the two spectra are identical in both peak height and width. The difference in A_m is quite small (<0.001), well within the limit of our experimental error (~10%). With $\rho = 1.0$, $E_x^2/E_y^2 = 0.12$, and $E_z^2/E_y^2 = 1.08$, we obtain $\theta_\mu = 41°$. Interestingly, the angle is close to $\theta_\mu = 37$ and 48° determined for cyt *c* on the surface of a silicon oxynitride (SiO_xN_y) waveguide using the techniques of TIR absorption and resonance Raman, respectively, with a monochromatic light source.[12b] The angle also lies in the range of $\theta_\mu = 34-45°$ obtained for H_2-cyt *c* (containing an iron-free heme) adsorbed to a quartz substrate using a TIRF technique.[22] In Figure 6b, we compare also the spectra of cyt *c* on the hydrophobic surface acquired using two different polarizations. Again, the two spectra are essentially identical, suggesting a mean tilt angle of $\theta_\mu = 41°$ as well. Notably, the angle also agrees satisfactorily with the orientation distribution of 48 ± 3° determined for cyt *c* on the silanized SiO_xN_y waveguide surface.[13b]

It is instructive to compare our result with the electrostatic properties of the investigated protein. The cytochrome *c* protein is basic, containing 19 lysines, 2 arginines and 12 acidic residues (aspartic or glutamic acids);[17,25] it is positively charged (+9) at neutral pH. Koppenol and Margoliash[34] have investigated the charged residues on the surface of horse heart cyt *c* and have found that the distribution of the charges is highly asymmetric, yielding a dipole of 325 D. The angle between the dipole vector and the heme plane is 33°. Hence, it is anticipated that when the protein is adsorbed to a negatively charged surface, such as that of the bare glass substrate at neutral pH, the angle between the heme plane and the surface would be approximately 33° if the adsorption is governed solely by electrostatic forces and the conformational change is insignificant. In this experiment, we determine a tilt angle of $\theta_\mu = 41°$, which deviates from the above angle by 8°. The deviation may arise from two major and, perhaps, competing factors: protein−surface and protein−protein interactions. The possibility of the protein−protein interaction, if there is any, can be tested by conducting a surface coverage dependence measurement.

Lee and Saavedra[13a] have explored the coverage dependence for Mb films adsorbed on the SiO_xN_y waveguide, measured as a function of bulk protein concentration after incubating the sample for 30 min. A sharp change of the mean heme tilt angle from ~70 to ~45° was found on a hydrophilic surface as the bulk concentration is raised above 5 μM. In contrast, the angle changes from ~20 to ~45° on the hydrophobic surface at nearly the same concentration range. For cyt *c*, a very different behavior was observed; within the limit of our experimental error, no significant changes in molecular orientation were observed when the bulk protein concentration was increased from 1 to 150 μM on both the hydrophilic and hydrophobic surfaces. The tilt angle remains at $\theta_\mu = 41°$. We are led to the conclusion that the molecular orientation of cyt *c* adsorbed to the silica surface is mainly determined by the protein−surface interaction and is little affected by the surrounding protein molecules. Therefore, the 8° deviation in the heme tilt angle, as discussed earlier, seems to indicate that localized interactions of the specific basic residues (such as lysines) in the protein with the negatively charged surface should be considered in understanding the molecular adsorption and orientation properties.[35]

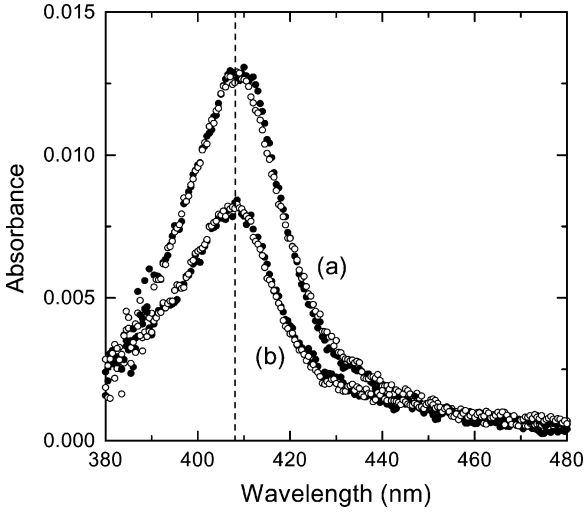

Figure 6. Polarization absorption spectra of cyt *c* on the (a) hydrophilic and (b) hydrophobic surfaces at the bulk concentration of 110 μM at pH 7.2 in 7 mM phosphate buffer. The spectra were acquired at the parallel (○) and perpendicular (●) polarizations. The dashed line denotes the wavelength $\lambda = 408$ nm.

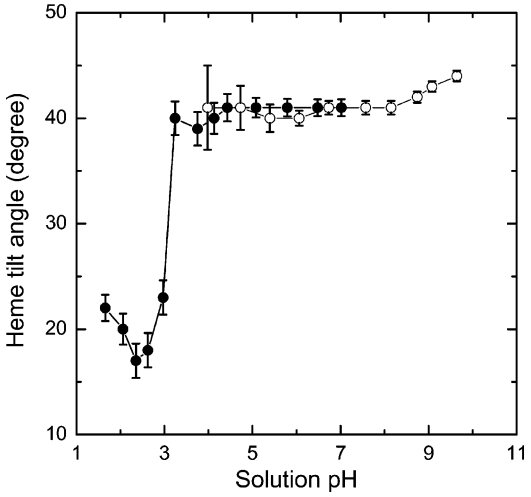

Figure 7. Changes of the mean heme tilt angle (θ_μ) of cyt *c* on both the hydrophilic (○) and hydrophobic (●) surfaces with solution pH. The protein concentration was fixed at 110 μM in 7 mM phosphate buffer.

Variations in pH may produce a more profound effect upon the molecular orientation.[36] This effect is anticipated because the adsorption of cyt *c* on the glass surface is predominantly governed by electrostatic forces;[13a,14] therefore, changing the charge states of both the substrate and the protein may lead to different adsorption geometry. Figure 7 shows the mean tilt angle of the heme plane as a function of bulk solution pH over the range of 1−9 on both the hydrophilic and hydrophobic surfaces. The corresponding spectra around pH 3 are given in Figure 8. Indeed, the orientation is seen to change sharply with solution acidity for cyt *c* adsorbed to the hydrophobic surface at pH ≈ 3, where the mean tilt angle decreases abruptly from $\theta_\mu = 41 \pm 2$ to 20 ± 3°. This kind of transition, however, cannot be clearly detected on the hydrophilic surface because the amount of cyt *c* adsorbed to the surface is very low in this pH

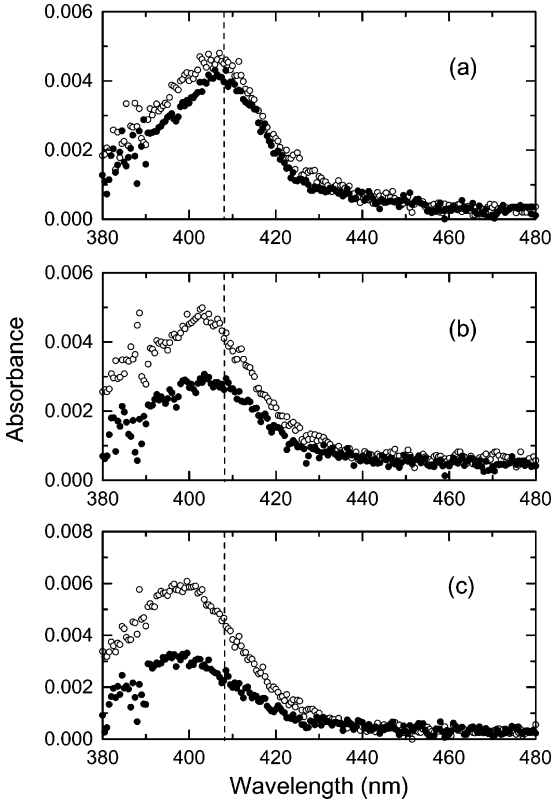

Figure 8. Polarization absorption spectra of cyt *c* adsorbed to the hydrophobic surface at pH values of (a) 3.8, (b) 3.0, and (c) 2.1. The spectra were acquired at the parallel (○) and perpendicular (●) polarizations. Note that the discontinuity appearing at ~390 nm is due to the change of the grating of the spectrometer and that the dashed lines denote the wavelength $\lambda = 408$ nm.

region (typically $A_m < 0.002$ in Figure 4b). One may associate this sharp transition with the conformational change of the protein, which unfolds at $pH_t \approx 3$ as clearly shown by the blue shift of the Soret band in Figure 8. The prevalence of the hydrophobic force,[37] which manifests itself at pH < 4 as discussed in the next section, may make an important contribution to the change of the tilt angle, too.

Packing Density. Cytochrome *c* is a globular protein with crytallographic dimensions of 2.5 × 2.5 × 3.7 nm.[38] Assuming that the conformational change is insignificant upon cyt *c* adsorption, the maximum packing density should range from 1.7×10^{-11} to 2.6×10^{-11} mol/cm^2, depending on the protein's orientation. This transforms to a packing density of $(1.0-1.6) \times 10^{13}$ molecules/cm^2 when a uniform monolayer of closely packed protein molecules forms on the surface. In determining the packing density by linear dichroism using an Ar ion laser, Lee and Saavedra[13a] considered with care that the molar absorptivities of protein in solution and on an integrated optical waveguide may not be the same if differences in the protein's orientation and/or conformation exist. This led them to take an alternative approach by determining the surface coverage using a pyridine hemochrome assay[39] for cyt *c* adsorbed to glass beads of 3 ± 0.3 mm diameter. Adsorbate densities of 2.9×10^{-11} mol/cm^2 on a hydrophilic surface and 1.7×10^{-11} mol/cm^2 on a hydrophobic surface were obtained. However, as frankly pointed out by the authors, this approach encountered a number of uncertainties in their measurements. One of them is clearly that the total surface area of the beads is not precisely known, which may give rise to an error of up to ±25%.

We attempt here to derive the adsorbate packing density (Γ) directly from the photometry[40,41] of the observed polarization spectra. For a protein film containing molecules with an isotropic distribution, Γ (in mol/cm^2) can be calculated as[12,32]

$$\Gamma = \frac{A_t - A_b}{1000(n_{21}\epsilon E_0^{\,2}/\cos\theta)N} \tag{12}$$

where $E_0^2 = E_x^2 + E_z^2$ and $E_0^2 = E_y^2$ are the squares of the electric field amplitude given at the parallel and perpendicular polarizations, respectively, and N is the number of internal reflections. However, when applied to describing a film composed of oriented molecules, this set of equations should be modified to

$$\Gamma = \frac{A_{t,\parallel} - A_{b,\parallel}}{1000[n_{21}(\epsilon_x E_x^{\,2} + \epsilon_z E_z^{\,2})/\cos\theta]N} \tag{13}$$

and

$$\Gamma = \frac{A_{t,\perp} - A_{b,\perp}}{1000(n_{21}\epsilon_y E_y^{\,2}/\cos\theta)N} \tag{14}$$

where ϵ_x, ϵ_y, and ϵ_z are the molar absorptivities of the oriented molecules along the *x*, *y*, and *z* axes, respectively, and are proportional to the squares of the transition dipole moments of the molecule in each direction, μ_x^2, μ_y^2, and μ_z^2. Following Fraaije et al.,[22] we consider the molar absorptivities (ϵ_x, ϵ_y, and ϵ_z) of the heme group for the surface-bound cyt *c* in terms of the two degenerate transition dipoles (a_{1u}, $a_{2u} \rightarrow e_g$) lying perpendicular to each other in the porphyrin plane (Figure 1b).[42] Assuming that the two dipoles are similar in magnitude (i.e., $|\mu_1| \approx |\mu_2| \approx \mu$), the authors showed that the time-averaged transition moments are $\mu_x^2 = \mu_y^2 = \mu^2(1 + \sin^2\theta_\mu)/4$ and $\mu_z^2 = \mu^2\cos^2\theta_\mu/2$. Notably, depending on θ_μ, these moments may differ markedly from the corresponding terms, $\mu_x^2 = \mu_y^2 = \mu_z^2 = \mu^2/3$, of the protein in solution. The factor of $^1/_3$ in the latter terms stems from the fact that the dipoles in the porphyrin ring of the solution of cyt *c* are randomly oriented with respect to either the parallel or the perpendicularly polarized light. At $N = 1$ and assuming that the conformational change is insignificant, we have

$$\frac{A_{t,\parallel} - A_{b,\parallel}}{1000} = \frac{3\Gamma n_{21}\epsilon_b}{4\cos\theta}[E_x^{\,2}(1 + \sin^2\theta_\mu) + 2E_z^{\,2}\cos^2\theta_\mu] \tag{15}$$

and

$$\frac{A_{t,\perp} - A_{b,\perp}}{1000} = \frac{3\Gamma n_{21}\epsilon_b}{4\cos\theta}[E_y^{\,2}(1 + \sin^2\theta_\mu)] \tag{16}$$

Both equations represent an extension of the calculations for the adsorbate with one transition dipole per molecule.[22,40,41]

With $\theta_\mu = 41°$ and the absorbance maximum of $A_m = 0.015 \pm 0.001$ in Figure 3, we obtain a packing density of $\Gamma = (2.3 \pm 0.2) \times 10^{-11}$ mol/cm^2 or $(1.4 \pm 0.1) \times 10^{13}$ molecules/cm^2 for cyt *c* on the hydrophilic surface at neutral pH. Note that this packing density is in good agreement with the value determined by Lee and Saavedra[13a,b] but is ~4 times larger than that originally reported by Walker et al.[12b] It is also noteworthy that this Γ is well within the saturation range of $(1.0-1.6) \times 10^{13}$ molecules/cm^2 expected from the protein size, suggesting that the protein molecules are packed rather closely on the surface, forming a uniform layer at pH 7.2 and $C_b = 110$ μM. Compared to this monolayer coverage, the packing density of the protein on the hydrophobic surface (Figure 3) is significantly

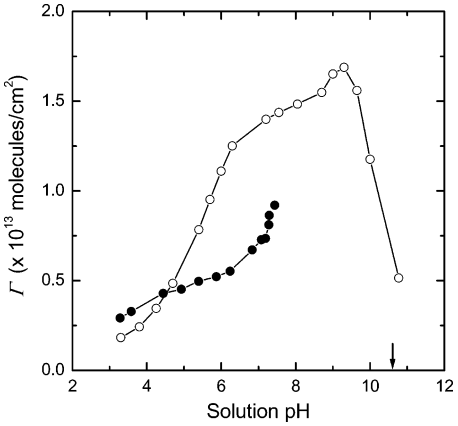

Figure 9. Changes in the packing density (Γ) of cyt *c* on the hydrophilic (○) and hydrophobic (●) surfaces with solution pH. The protein concentration was fixed at 110 μM in 7 mM phosphate buffer. The arrow indicates the isoelectric point of cyt *c* at pI = 10.6.

lower, 7.8×10^{12} molecules/cm^2, meaning that only half of the surface is covered by the cyt *c* molecules.

Figure 9 shows the pH dependence of cyt *c* adsorption to both the hydrophilic and hydrophobic surfaces. The measurements were conducted over pH ranges of 3−11 for the hydrophilic substrate and 3−8 for the hydrophobic substrate, over which the protein retains nearly the same conformation. The error in this Γ determination is roughly ±10% after proper calibration of the measured A_m against ϵ_b, which in turn is pH-dependent (cf. Figure 4a). On the bare surface, the packing density is observed to increase dramatically with solution pH in the acidic region and reach the maximum at pH 9.5, where the surface is completely covered by the cyt *c* molecules. At the two extreme regions, pH < 5 or pH > 10, the Soret band intensity diminishes rapidly. The result correlates well with the isoelectric points of the protein and the surface (pI = 10.6 for cyt *c* and p$I \approx 3$ for the silica substrate)[43,44] (i.e., at pH < 3, the adsorption of the basic protein to the positively charged glass surface is not energetically favored, and at pH > 10, both the protein and the surface are negatively charged and the interaction is repulsive). It is also consistent with the existing large body of evidence[13a,14,29,44] that the adsorption of cyt *c* to the bare glass surface is predominantly governed by electrostatic forces.

The strong pH dependence of protein adsorption to the hydrophilic surface may be compared to that reported by Kondo et al.,[45] who studied the binding of cyt *c* to silica colloids of 0.12-μm diameter. Instead of directly measuring the Soret band intensity of the adsorbed cyt *c* molecules, the authors deduced the adsorption quantity by determining the reduction of the bulk protein concentration after adding the colloid suspension to the solution. Their result, similar to ours (cf. Figure 9), shows that the amount of cyt *c* adsorbed to the hydrophilic surface is highest at pH ≈ 8.5 but decreases markedly at pH ≈ 10.[45b] The behavior again is understandable from the viewpoint that at pH 9.5 the net charge on cyt *c* is nearly zero and thereby the intermolecular repulsion between the adsorbed molecules is minimal, leading to the preferential adsorption of the protein to the surface. The maximum packing density they determined at pH 9.5 is $\Gamma = 1.4 \times 10^{13}$ molecules/cm^2, which is close to our measurement of $\Gamma = 1.6 \times 10^{13}$ molecules/cm^2 (cf. Figure 9).

It should be noted that in Figure 9 the protein adsorption shows markedly different behavior on both the hydrophilic and hydrophobic surfaces as a function of solution pH. As a result, there is a curve crossing at pH ≈ 4.5 between these two cases. At pH > 5, the amount of cyt *c* adsorbed to the hydrophilic surface is higher than that on the hydrophobic surface, but it becomes significantly lower at pH < 4. Because at pH ≈ 3 the electrostatic interaction between the protein and the surface on average is repulsive, the observation that more protein molecules are adsorbed to the silanized surface than to the bare surface indicates that hydrophobic forces, in addition to electrostatic forces, also play an important role in the adsorption process. The hydrophobic forces may even dominate the adsorption in the lower-pH region for the silanized substrate. The importance of the hydrophobic interactions in protein adsorption has been emphasized by Gast and co-workers[37] for ribonuclease A on polystyrene surfaces.

Conclusions

The Soret band is a sensitive probe of adsorption equilibrium, molecular orientation, packing density, and conformational changes of heme proteins bound to a fused silica surface. It is a direct and promising approach for further examination of protein adsorption on a single-crystal surface,[40,41,46] on which the molecular orientation of a protein is expected to be determined more precisely using linearly polarized light. The method can be applied to the study of other heme-containing proteins (such as myoglobin and hemoglobin)[47] on the surfaces of any other optically transparent substrates as well.

The adsorption of cyt *c* on the bare fused silica surface depends strongly on bulk solution pH but shows only a weak dependence on the same surface after silanization. The result is consistent with the picture that protein adsorption on the hydrophilic glass surface at neutral pH is governed by electrostatic forces. The electrostatic interaction, however, is greatly lessened on the silanized surface, where the hydrophobic force prevails at pH < 4. This suggests that the Soret absorption band can be used as a sensitive probe of the hydrophobicity of a surface under investigation.

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