Molecular Orientation Distributions in Protein Films. 2. Site-Directed Immobilization of Yeast Cytochrome *c* on Thiol-Capped, Self-Assembled Monolayers

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Abstract: Molecular orientation in films of yeast cytochrome *c* immobilized via disulfide bonding between cysteine 102 and the thiol tail groups of self-assembled monolayers (SAMs) coated on planar glass substrates was investigated. The orientation distribution of the heme groups in the protein film was determined using a combination of absorption linear dichroism, measured in a planar integrated optical waveguide-attenuated total reflection geometry, and emission anisotropy, measured in a total internal reflection fluorescence geometry. The mean heme tilt angle and angular distribution about the mean were recovered using a Gaussian model for the orientation distribution. These data are the first orientation distribution measurements reported for a protein film immobilized using a site-directed bonding strategy. The results show that the molecular architecture examined in this study does not produce a highly oriented protein film. A significant fraction of the immobilized cytochrome *c* is nonspecifically adsorbed to the SAM surface, which produces a relatively broad distribution of heme orientations.

Introduction

Preparation and characterization of protein films immobilized at the interface between a synthetic, insoluble substrate and a liquid phase is a topic of widespread interest in both academic and industrial research laboratories. This interest stems from the current widespread use of these films in bioassays and affinity-based separations, and their potential use in molecular device technologies such as bioanalytical sensing and energy storage/conversion.[1−3]

Since the spatial distribution of ligand binding sites over the surface of a protein is typically asymmetric, the geometric orientation of an immobilized protein molecule may determine if its native bioactivity is retained in the interfacial environment. For example, an antibody immobilized with the antigenic sites facing the substrate will be sterically restricted from binding a macromolecular antigen.[4] Consequently, the development of general methodologies to immobilize proteins in defined geometric orientations has been a focus of considerable research efforts in recent years.[4−17] The general approach has been to use a structurally unique site or region on the surface of the protein to geometrically "direct" the attachment of the molecule to an appropriately derivatized substrate surface.

Substantial work in this area has been performed using the high-affinity binding interaction between streptavidin and biotin, confined to an air−water interface, as a model system. For example, Ringsdorf and co-workers showed that a crystalline monolayer of streptavidin forms when the protein binds to a floating Langmuir monolayer doped with a biotin-capped phospholipid.[5] Electron crystallography was used to establish the orientation of the protein molecules in the film.[6] Biospecific adsorption was subsequently used to form mono- and multilayer assemblies containing other types of proteins (e.g., antibodies) at the air−water interface.[7]

The biotin−streptavidin architecture[8,9] and other types of biospecific adsorption[10,11] have been used to assemble mono- and multilayer protein films at solid−liquid interfaces. Other strategies that have been employed include: (i) physical adsorption or ligation via a unique functional group or chemically distinct "patch" on the surface of a protein to a chemically complementary substrate surface[12−15] and (ii) covalent bonding of a specific functional group on the surface of a protein to a pendant group on the substrate surface.[16,17] However, assess-

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ments of whether these methods actually produce oriented arrays of protein molecules are few. This shortcoming is attributable to a lack of analytical techniques appropriate for characterizing molecular orientation and macroscopic order in *noncrystalline* protein films at solid−liquid interfaces (i.e., films ranging from liquid crystalline to randomly oriented). Furthermore, to discriminate against artifacts, an approach that does not require the sample to be dried is preferred. Some progress has been made using polarized spectroscopic techniques,[16,18−21] X-ray interferometry,[14,17] fluorescence quenching,[12] and ligand binding methods.[10] With some of these techniques, the mean molecular orientation of a spectral probe in a protein film can be measured. Although useful, knowledge of the mean orientation is not sufficient to assess macroscopic order (and in order to extract a mean tilt angle, a narrow orientation distribution must be assumed). Synthetic strategies designed to produce oriented protein films could be more accurately evaluated using an analytical technique capable of also determining the *distribution* of molecular orientations in the film.[20,22]

An experimental technique for measuring the orientation distribution of porphyrin planes in a hydrated heme protein film supported on a solid substrate is described in the accompanying paper.[23] In this study, we employed this technique to examine a site-directed, covalent bonding strategy for oriented protein deposition. This is the first study of orientation distribution in a covalently immobilized protein film. Yeast cytochrome *c* was immobilized on thiol-capped, self-assembled monolayers (SAMs) formed from an alkyltrichlorosilane coated on planar glass substrates. In this architecture, immobilization (presumably) occurs via disulfide bonding between the single reduced cysteine on the protein and the thiol tail group on the SAM. The results show that this molecular architecture does not produce a highly oriented protein film. A significant fraction of the immobilized cytochrome *c* molecules are nonspecifically adsorbed to the SAM surface, which produces a relatively broad distribution of heme orientations. From a more general perspective, the results illustrate that, in order to use a site-directed, specific protein−substrate interaction to create an oriented protein film, nonspecific interactions must be minimized.

Experimental Section

Surface Preparation and Characterization. Silicon oxynitride planar waveguides were used as substrates for linear dichroism experiments.[24] Fused silica (Dynasil, Berlin, NJ) and fused quartz (Hereaus Amersil, Duluth, GA) slides were used as substrates for total internal reflection fluorescence (TIRF) anisotropy experiments.

The surfactant 1-(thioacetato)-16-(trichlorosilyl)hexadecane was used to prepare substrates coated with SAMs bearing a thioacetate tail group, followed by *in situ* reduction to a thiol tail group, as described in the accompanying paper.[23] Thioacetate and thiol SAMs were also prepared on silicon wafers and Si attenuated total reflection (ATR) crystals, under conditions identical to those used for waveguide and TIRF substrates. Wafers and crystals were cleaned by treatment for 30 min in an argon plasma (Harrick PDC-3XG). FT-IR spectroscopy was used to monitor conversion of the thioacetate SAM to the thiol SAM. Spectra were measured in an ATR geometry, using the uncoated, oxidized Si crystal to acquire the reference spectrum. The characteristic carbonyl band of the thioacetate at 1741 cm^{-1} disappeared after reduction with $LiAlH_4$, showing essentially quantitative conversion to the thiol (spectra not shown). Ellipsometry was performed on thiol SAMs deposited on Si wafers, as described previously.[22] The measured thickness was 26 ± 1.5 Å ($n = 3$), using a refractive index of 1.46 for the SAM.

The chemical availability of sulfhydryl groups on SAM-coated substrates was semiquantitatively assessed by reacting substrates with 5-[[2(and 3)-(*S*)-(acetylmercapto)succinoyl]amino]fluorescein (SAMSA fluorescein; Molecular Probes, no. A-685).[25] Substrates were incubated with 150 μM SAMSA fluorescein (activated according to the supplier's instructions) in 200 mM phosphate buffer, pH 7.3, containing 200 mM NaCl, for 60 min, followed by rinsing in buffer. Epifluorescence microscopy was used to monitor the relative surface coverage of SAMSA fluorescein on thiol SAM-coated substrates.

Protein Solutions. Wild-type yeast iso-1-ferrocytochrome *c* from *Saccharomyces cerevisiae* (cyt *c*) was obtained from Sigma (no. C 2436) and used as received. Solutions were prepared in 50 mM phosphate buffer, pH 7.2. Assaying the protein solution using 5,5′-dithiobis(2-nitrobenzoic acid)[26] showed that cysteine 102 was nearly 100% monomeric when solutions were used immediately after preparation. However, when found to be necessary, solutions that were stored for later use were treated with dithiothreitol to reduce dimers and purified on a Sephadex G-25 column. Zinc-substituted yeast cytochrome *c* (Zn-cyt *c*) was prepared using the procedure described for zinc-substituted horse heart cytochrome *c* in ref 23. Protein concentrations were determined using $\epsilon_{416\ nm} = 96\ 000\ M^{-1}\ cm^{-1}$ for cyt *c* (our measurement) and $\epsilon_{423\ nm} = 243\ 000\ M^{-1}\ cm^{-1}$ for Zn-cyt *c* (which is the published value[27] for zinc-substituted horse heart cytochrome *c*).

Immobilized Protein Films. Cyt *c* films were immobilized on thiol SAM-coated, planar substrates by soaking the substrate in a 35 μM protein solution for either 8 or 48 h at room temperature in a sealed container. For 8 h incubations, the ionic strength of the protein solution was increased by adding 100 mM NaCl. For linear dichroism measurements, the incubation was performed with the waveguide mounted in a flow cell. For TIRF measurements, the solution contained a 1:8 molar ratio of Zn-cyt *c*/cyt *c*. The Zn-cyt *c* was diluted with cyt *c* to prevent energy transfer between protein molecules in the film.[23] After incubation, substrates were rinsed in phosphate buffer, without allowing the film to dry, prior to commencing spectral measurements. For 8 h incubations, the rinse buffer contained 100 mM NaCl.

Cyt *c* was also immobilized on planar substrates using a published method for antibody immobilization.[28] The hydrophilic substrate was soaked for 2 h in a 2% (v/v) solution of (3-mercaptopropyl)-trimethoxysilane (MPTS; Sigma) in anhydrous toluene (distilled over sodium), which generated a surface derivatized with sulfhydryl groups. After being rinsed sequentially with toluene, acetone, and water and dried under a N_2 stream, the substrate was treated with γ-maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS), which functioned as a cross-linker between the sulfhydryls on the substrate and lysine amino groups on the protein. After a 1 h incubation in 5 mM GMBS solution (dissolved in *N*,*N*-dimethylformamide and diluted with absolute ethanol), the substrate was washed with phosphate buffer and then soaked in a 50 μM solution of cyt *c* in phosphate buffer (cyt *c* for waveguide substrates, a 1:8 molar ratio of Zn-cyt *c*/cyt *c* for TIRF substrates). Protein solutions were incubated for 2 h before the substrate was rinsed with phosphate buffer. For linear dichroism measurements, the protein immobilization step was performed with the waveguide mounted in the flow cell.

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TIR Spectroscopies. The procedures used to perform IOW-ATR (IOW = integrated optical waveguide) linear dichroism and TIRF anisotropy measurements were essentially identical to those described in the accompanying paper.[23] In linear dichroism experiments, the blank propagation loss coefficients of the waveguide in both polarizations were measured with buffer in the flow cell. After the protein solution was injected and allowed to incubate for the prescribed period, the flow cell was flushed with buffer and the propagation loss coefficients were measured again. The loss coefficients due solely to the immobilized protein film were then recovered by difference. Orientation distributions ($\theta_\mu \pm \theta_o$) were calculated as described in the accompanying paper,[23] assuming a value of 41° for γ.

Protein Surface Coverages. Surface coverages were measured for cyt *c* immobilized on thiol SAMs using an approach similar to that employed for adsorbed horse heart cyt *c* in the accompanying paper.[23] However, no single desorption treatment was found to be capable of quantitatively removing the protein from these substrates. Surface coverages were therefore measured by a pair of complementary absorbance and fluorescence experiments. In the absorbance assay, the absolute amount of protein removed from the substrate using a specific desorption treatment was measured. The fluorescence assay was used to determine what fraction of the total amount of adsorbed protein was removed from the substrate under identical desorption conditions. The surface coverage was then calculated from the ratio of the absolute amount desorbed (in monolayer units) to the fraction of the total amount adsorbed.

Two different desorption conditions were used in both the absorbance and fluorescence assays: phosphate buffer containing 100 mM NaCl, and phosphate buffer containing 100 mM NaCl and 5 mM dithiothreitol (DTT). Thiol SAM-coated glass beads were used as the substrate for the absorbance assays due to their high surface area to volume ratio. The beads were silanized, and cyt *c* was deposited under conditions identical to those used for preparing samples for linear dichroism measurements. The amount of desorbed protein was calculated using the molar absorptivity of the native protein. Fused silica and fused quartz substrates were used for the fluorescence assays. Preparation of thiol SAMs and protein deposition were performed under conditions identical to those used for preparing samples for TIRF anisotropy measurements. Emission intensities were measured on an epifluorescence microscope.

Given the assumptions and uncertainties inherent in this experimental approach (which are discussed in refs 4 and 23), the surface coverage measurements should be considered approximate. Surface coverage was not measured for cyt *c* deposited on GMBS-derivatized substrates.

Results and Discussion

The primary goal of this study was to examine the hypothesis that a covalently immobilized protein film having a defined macroscopic orientation can be produced using site-directed bonding between a unique functional group near the surface of the protein and an appropriately derivatized substrate surface. This study makes use of our development of an experimental approach for measuring the orientation distribution of porphyrin planes in a hydrated protein film supported on a solid substrate, as described in the accompanying paper.[23]

The components of the molecular architecture chosen to address the hypothesis are diagrammed in Figure 1. Cyt *c* is immobilized on a SAM formed from a thiol-capped, hexadecyltrichlorosilane coated on a planar glass substrate. Cyt *c* from yeast (wild-type, iso-1) was chosen because it has a single cysteine at position 102 that can form a disulfide bond with an extrinsic sulfhydryl group.[29] This cysteine therefore provides a geometrically defined site for attaching the protein via disulfide bonding to a thiol-functionalized substrate surface. This architecture is essentially identical to that used by Amador et al.[17a] and Chupa et al.[17b] in their X-ray interferometry studies of proteins immobilized to SAMs.

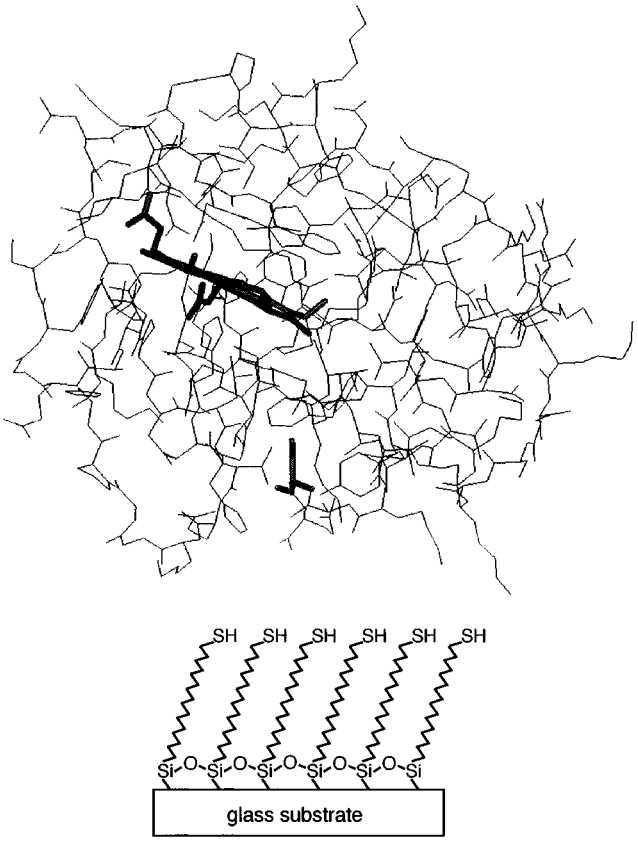

Figure 1. Films of yeast iso-1-cytochrome *c* were formed by depositing the protein (top) on SAMs (bottom) prepared from a thioacetate-capped, hexadecyltrichlorosilane coated on glass and quartz substrates. The thioacetate was reduced to the thiol prior to protein deposition. The heme group and cysteine 102 are highlighted in the protein structure.

Protein Surface Coverages. The results of the surface coverage assays for cyt *c* films, expressed in monolayer units, are listed in Table 1. These data are based on a coverage of 2.2×10^{-11} mol/cm^2 as the equivalent of one monolayer, which assumes that the orientation of the molecules in the film is geometrically random and no "spreading" occurs due to adsorption-induced conformational changes.[18c] Deposition from a solution containing 35 μM protein and 100 mM NaCl for a period of 8 h produced a surface coverage of about 0.3 monolayer. In an effort to increase the coverage, the deposition was performed for 48 h from a 35 μM protein solution that did not contain added NaCl. These differences produced an increase in coverage to approximately one monolayer. It is not known if the increased surface coverage was due to the longer deposition time, the lower ionic strength, or both.

Orientation Distributions. The results of IOW-ATR linear dichroism and TIRF anisotropy measurements performed on immobilized films of cyt *c* are listed in Table 1. The orientation distribution of the porphyrin planes in the film deposited on the thiol SAM for 8 h was 67° ± 39°. From a qualitative examination of the crystal structure of cyt *c* (Figure 1), the angle between the face of the protein on which cysteine 102 is located and the molecular plane of the heme appears to be in the range of 40−65°, depending on how the cysteine side chain is "unwound" to allow disulfide bonding to the thiol SAM.[30] Thus, the θ_μ value of 67° for the 8 h film is not unreasonable, assuming that the protein is immobilized as suggested in Figure 1.

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Table 1. Surface Coverages and Orientation Distributions for Immobilized Cytochrome *c* Films

substrate coating	surface coverage[a] (monolayers)	dichroic ratio (ρ)	anisotropy (r)	orientation distribution ($\theta_\mu \pm \theta_\sigma$, deg)
thiol SAM (8 h deposition time)	0.3	1.27 ± 0.38 ($n = 3$)	-0.163 ± 0.014 ($n = 3$)	67 ± 39
thiol SAM (48 h deposition time)	1	1.35 ± 0.02 ($n = 2$)	-0.103 ± 0.014 ($n = 5$)	n/o[b]
GMBS/MPTS	n/m[c]	1.05 ($n = 1$)	-0.129 ± 0.019 ($n = 3$)	45 ± 23

[a] Based on one monolayer $= 2.2 \times 10^{-11}$ mol/cm^2. [b] Not obtainable (an orientation distribution could not be calculated due to the physical inconsistency between the measured values of ρ and r, assuming a Gaussian distribution model). [c] Not measured.

Table 2. Orientation Distributions (deg) Calculated for Selected Combinations of Emission Anisotropy and Dichroic Ratio: Application to Cyt *c* Films Deposited on Thiol SAMs for 48 h[a]

	anisotropy (r)				
dichroic ratio (ρ)	-0.089	-0.103 ± 0.014[b]	-0.117	-0.125	-0.131
1.30	n/o[c]	n/o	43 ± 10	43 ± 11	43 ± 18
1.32	n/o	n/o	n/o	43 ± 6	45 ± 13
1.35 ± 0.02[b]	n/o	n/o	n/o	n/o	44 ± 10
1.38	n/o	n/o	n/o	n/o	44 ± 7
1.40	n/o	n/o	n/o	n/o	43 ± 3

[a] Gaussian orientation distributions expressed as $\theta_\mu \pm \theta_\sigma$. [b] Measured ρ and r values (mean $\pm$ standard deviation) for cyt *c* films deposited on thiol SAMs for 48 h. [c] Not obtainable (the combination of ρ and r did not produce a simultaneous solution to eqs 1 and 8 in the accompanying paper.[23]).

For the cyt *c* films formed on thiol SAMs during a 48 h deposition period, an angular distribution could not be calculated from the pair of measured mean parameters ($\rho = 1.35$ and $r = -0.103$). In other words, these two parameters are physically inconsistent with a Gaussian distribution model for a circularly polarized oscillator with $\gamma = 41°$. As discussed in the accompanying paper,[23] the cause(s) of this inconsistency are unknown.

However, orientation distributions could be calculated for other pairs of ρ and r that were within 2 standard deviations of the respective mean values, as shown in Table 2. The orientation distributions listed there have θ_μ values that range from 43° to 45° and θ_σ values ranging from ±3° to ±18°. The wide range of angular distributions prevents us from establishing a "representative" value for the orientation distribution, as done in the accompanying paper.[23] In other words, an angular distribution of ±3° represents a highly ordered film, whereas ±18° represents a moderately disordered film.[31] Despite our inability to assign an orientation distribution to the 48 h film, it is apparent from the difference in measured anisotropies (see Table 1) that the 48 h film is structurally distinct from the 8 h film.

Another type of protein film, cyt *c* immobilized on GMBS-derivatized substrates, was also examined. In this film, the protein is (presumably) immobilized via covalent bond formation between its lysine amino groups and substrate-bound succinimide moieties. From the dichroic ratio and anisotropy measurements, an orientation distribution of $45° \pm 23°$ was calculated (all data are listed in Table 1). The width of this distribution is therefore substantially less than the distribution of ±39° determined for 8 h films deposited on thiol SAMs, which indicates a relatively higher degree of order on the GMBS-coated substrates. The difference is illustrated in Figure 2, where the respective normalized orientation distributions are plotted. For comparison purposes, the distribution for an arachidic acid Langmuir−Blodgett (LB) film doped with an amphiphilic zinc porphyrin (see the accompanying paper[23]) is also plotted.

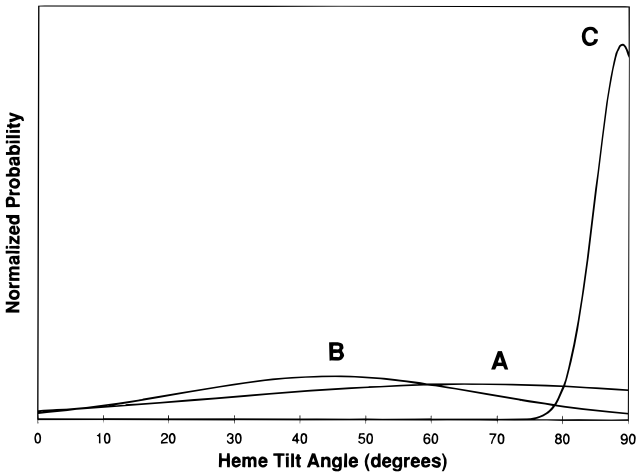

Figure 2. Gaussian probability distributions for (A) cyt *c* immobilized on thiol SAM with 8 h deposition, $\theta_\mu = 67°$ and $\theta_\sigma = \pm 39°$, (B) cyt *c* immobilized on GMBS/MPTS, with $\theta_\mu = 45°$ and $\theta_\sigma = \pm 23°$, (C) Zn-TOPP doped into a LB bilayer of arachidic acid with $\theta_\mu = 89°$ and $\theta_\sigma = \pm 4°$ (from ref 23).

Given the asymmetric distribution of lysine residues over the surface of cyt *c*,[29] it is not surprising that a film with only a moderate degree of disorder is produced when a lysine-directed immobilization method is employed. However, it is surprising that protein films formed on thiol SAM-coated substrates are considerably more disordered, since reduced thiol groups occur much less frequently than amino groups in cyt *c*.[29] This apparent contradiction is discussed below.

The θ_σ of ±39° for 8 h yeast cyt *c* films is also substantially greater than some of the angular distributions reported for horse heart cyt *c* films in the accompanying paper.[23] In that study, θ_σ values near ±10° were determined for (approximately) one monolayer of horse heart cyt *c* adsorbed to Langmuir−Blodgett films of arachidic acid and to glass derivatized with dichlorodimethylsilane. Films adsorbed on three other types of substrates were relatively disordered ($\theta_\sigma \geq \pm 20°$). In both cases where a narrow orientation distribution was present, a single type of noncovalent interaction appeared to dominate the adsorptive interaction between the surfaces of the protein and the substrate.

Protein−SAM Interactions. One would expect that if disulfide bonding was the primary interaction between the surfaces of cyt *c* and the thiol-capped SAM, the distribution would be relatively narrow since there is only one available cysteine on the protein. Furthermore, if disulfide bonding was the primary interaction, treatment with a disulfide reducing agent would cause the majority of the immobilized protein to desorb.

Consequently, a limited investigation into the physical nature of cyt *c* binding to thiol SAMs was undertaken. Epifluorescence microscopy of Zn-cyt *c* was used to quantitatively monitor the extent of protein desorption effected by soaking the film in

(31) Also note that the apparent discontinuity (i.e., "waist") in the response surface plotted in Figure 3a of the accompanying paper[23] occurs when r is -0.0944. This point corresponds to $\beta_\mu = 54.7°$ (or $\theta_\mu = 35.3°$), which is the mean tilt angle expected for a random Gaussian orientation distribution of circularly polarized oscillators with $\gamma = 41°$. When θ_μ is near 35°, it is difficult to use simultaneous measurements of ρ and r to determine θ_σ.

Table 3. Desorption of Cyt *c* from Thiol SAM-Coated Substrates in Salt, Dithiothreitol, and Surfactant Solutions[a]

desorption treatment applied after 8 h protein deposition	percentage (%) of initial fluorescence intensity remaining after applying desorption treatments
saline buffer rinse (50 mM phosphate, pH 7.2, containing 100 mM NaCl)	100[b]
saline buffer containing 200 mM KCl; 30 min static incubation	58 ± 7 ($n = 7$)
saline buffer containing 200 mM KCl + 5 mM DTT; 30 min static incubation	47 ± 13 ($n = 4$)
saline buffer containing 200 mM KCl + 2% Triton X-100 (v/v); 30 min static incubation	22 ± 4 ($n = 3$)

desorption treatment applied after 48 h protein deposition	percentage (%) of initial fluorescence intensity remaining after applying desorption treatments
buffer rinse (50 mM phosphate, pH 7.2)	100[c]
buffer containing 200 mM KCl; 30 min static incubation	66 ± 11 ($n = 8$)
buffer containing 200 mM KCl + 5 mM DTT; 30 min static incubation	64 ± 9 ($n = 4$)
buffer containing 200 mM KCl + 2% Triton X-100 (v/v); 30 min static incubation	52 ± 10 ($n = 4$)

[a] Films were formed by adsorption from solutions containing 1:8 Zn-cyt *c*/cyt *c*, under the same conditions used to form films for orientation distribution measurements (see the text). Epifluorescence emission intensities measured after application of each desorption treatment were normalized to the first value measured for each film after the initial rinse in phosphate buffer. [b] Surface coverage of ca. 0.3 monolayer. [c] Surface coverage of ca. 1.0 monolayer.

buffer solutions containing a high salt concentration, DTT, or a nonionic surfactant. The intent was to determine what fraction of the protein film was immobilized via disulfide bonding to the thiol SAM, and to explore possible causes for the surprisingly wide angular distribution of ±39° measured for the 8 h films.

For these experiments, the substrate was first mounted in a liquid cell. Protein films were formed by adsorption from solutions containing 1:8 Zn-cyt *c*/cyt *c*, under the same conditions used to form films for TIRF anisotropy measurements. After the film was rinsed in buffer containing 100 mM NaCl (saline buffer, ionic strength of 213 mM) without being allowed to dry, the cell was refilled with saline buffer and the fluorescence emission intensity was measured. The cell was then filled with saline buffer containing 200 mM KCl (ionic strength of 413 mM). After a soaking period of 30 min, the cell was refilled with saline buffer and the emission intensity was again measured. The same procedure was used to measure the effect of soaking the film in (i) saline buffer containing 200 mM KCl and 5 mM DTT and (ii) saline buffer containing 200 mM KCl and 2% (v/v) Triton X-100. Listed in Table 3 are the emission intensities measured after application of each desorption treatment, normalized to the first value measured for each film after the initial rinse in saline buffer.

The data indicate that the nature of the interaction between cyt *c* and the thiol SAM is complex. Some fraction of the protein molecules in the 8 h film appear to be electrostatically adsorbed, since treatment with high ionic strength buffer for 30 min desorbed 42% of the protein. In retrospect, this result is not surprising since it is well known that deposition of a silane monolayer on a glass surface reduces but does not eliminate its intrinsic negative charge.[32] However, it is somewhat surprising that the addition of 5 mM DTT removed very little additional protein beyond that desorbed in high ionic strength buffer. This result shows that little if any of the protein molecules are immobilized on the SAM surface *solely* through disulfide bonding. Soaking the film in high ionic strength buffer containing 2% Triton X-100 desorbed about 78% of the protein from the SAM surface. For the purposes of this discussion, the remaining 22% is considered "irreversibly" adsorbed. This result strongly implicates hydrophobic interactions as a major contributor to the forces that immobilize cyt *c* to thiol SAMs. For each of the desorption treatments, extending the soaking period to 24 h did not result in additional protein removal beyond that observed at 30 min.

Desorption experiments were also performed on cyt *c* films formed by 48 h deposition on thiol SAMs. The experimental procedure was identical to that described above, except that the buffer rinse did not contain 100 mM NaCl (since the protein deposition solution also did not contain added NaCl). The results, listed in Table 3, were similar to those observed for the 8 h films. The only significant difference is that 50% of the protein film was removed by soaking in the 2% surfactant solution, leaving 50% irreversibly adsorbed.

The lack of protein desorption observed when protein films were incubated with DTT raises the question of chemical availability of the thiol tail groups on the SAM. This issue was assessed by reacting thiol SAM-coated substrates with SAMSA fluorescein (SF), which forms disulfide bonds with reduced thiols.[25] SAMs formed using octadecyltrichlorosilane (OTS) were used as a control to assess the extent of nonspecific adsorption, and epifluorescence microscopy was employed to monitor SF adsorption and desorption from SAMs. The extent of SF binding to thiol SAMs was 15-fold greater than on OTS SAMs, indicating that specific chemisorption predominated over nonspecific physisorption. Soaking SF-treated, thiol SAMs in buffer containing 2 mM DTT for 1 h caused 50% of the surface-bound SF to desorb. From these results, we conclude that a significant fraction of the SF adsorbed on thiol SAMs was immobilized via disulfide bonding. Although these experiments do not provide information on the percentage of reactive thiol groups on the SAM, they do demonstrate that at least some of the thiols are accessible to a sulfhydryl-reactive molecule. In this context, it is important to note that on the basis of the projected areas (onto the $x-y$ plane) of a $C_{16}SH$ monomer and cyt *c*, less than 5% of the tail groups on the thiol SAM must be accessible to enable a close packed monolayer of cyt *c* to be chemisorbed via disulfide bonding.

It is clear from the data listed in Table 3 that several types of interactions exist between the surfaces of cyt *c* and thiol SAM-coated glass. In other words, a substantial fraction of the immobilized protein molecules are nonspecifically adsorbed in both the 8 and 48 h films. (Here the term "nonspecific" is used to refer to any protein−SAM interaction other than pure disulfide bonding at cysteine 102). This result is not surprising given the well-known tendency of many proteins to adsorb to virtually any surface.[30] It is possible that the addition of DTT *did* reduce disulfide bonds formed between the Cys 102 and the thiol SAM. However, since the DTT treatment did not cause significant desorption, the protein must be immobilized by forces other than (or in addition to) disulfide bonding. The substantial degree of nonspecific adsorption is one probable cause of the relatively broad angular distribution of ±39° measured for the 8 h film. In this scenario, the protein interacts with the thiol SAM surface in a variety of geometric orientations, generating a broader orientation distribution than expected on the basis of the idealized geometry depicted in Figure 1.

(32) See for example: Chen, M; Cassidy, R. M. *J. Chromatogr.* **1992**, *602*, 227−234.

Conformational change is a second possible cause for the broad orientation distribution in the 8 h film. In this scenario, a substantial fraction of the immobilized protein molecules undergo adsorption-induced conformational changes.[33] If these changes perturb the geometric relationship between the heme plane and polypeptide matrix that surrounds it, and if the degree of perturbation varies among the molecules, a broad molecular orientation distribution would result. Furthermore, the occurrence of a conformational change would probably lead to formation of additional, nonspecific adsorptive contacts between the protein and the substrate.[33] It is unlikely that reduction with DTT would be sufficient to quantitatively desorb molecules that were both specifically and nonspecifically bound. In a very recent paper, Tobias et al.[34] used molecular dynamics simulations to study the conformation of a yeast cyt *c* molecule disulfide bonded to a thiol SAM. Their results predict that the protein undergoes minor structural changes when it partially "dissolves" into the SAM (i.e., the polar side chains of the protein extend to "wet" the SAM surface). Thus, tethering the protein to the SAM is predicted to result in the formation of additional, noncovalent binding interactions.

However, to date we have no experimental data that describe the conformational state of cyt *c* immobilized on thiol SAMs. Therefore, at this point we cannot determine if the orientational disorder in the 8 h film is due to (i) a substantial degree of nonspecific protein adsorption, which produces a broad distribution of geometric orientations, (ii) a considerable variation in the extent of conformational change among adsorbed protein molecules, which also results in a broad distribution of geometric orientations, or (iii) a combination of (i) and (ii).

(33) The tendency of proteins to undergo conformational changes upon adsorption to an interface is well recognized, but poorly understood from a fundamental perspective. See: (a) Andrade, J. D.; Hlady, V. *Adv. Polym. Sci.* **1986**, *79*, 1−63. (b) Haynes, C. A.; Norde, W. *Colloids Surf. B: Biointerfaces* **1994**, *2*, 517−566. (c) Brash, J. L.; Horbett, T. A. In *Proteins at Interfaces II*; Horbett, T. A., Brash, J. L., Eds.; ACS Symposium Series 602; American Chemical Society: Washington, DC, 1995; pp 1−23 and references therein.

(34) Tobias, D. J.; Mar, W.; Blasie, J. K.; Klein, M. L. *Biophys. J.*, in press.

Conclusions

Developing an experimental approach designed to immobilize a macroscopically oriented protein film at a solid−liquid interface consists of two major steps: (1) devising an experimental strategy and using it to construct the molecular assembly and (2) evaluating if the experimental strategy successfully produced the molecular assembly that was envisioned. Numerous studies have addressed the first step.[4−17] However, due to the difficulty of measuring molecular orientation in hydrated protein films, few groups have attempted to address the second step. The results reported here are the first direct measurements of molecular orientation distribution in a hydrated protein film immobilized using a site-directed bonding strategy.

Overall, our results show that the molecular architecture selected for this initial study, yeast iso-1-cyt *c* disulfide bonded to a thiol-capped SAM, does not produce a highly oriented film. A significant fraction of the immobilized protein molecules nonspecifically interact with the SAM, which probably contributes to the relatively broad orientation distribution of heme groups in the film. The extent to which nonspecific protein−substrate interactions compete with the desired, specific interaction is certainly a key issue for production of protein films where orientation is an important consideration. Lastly, from a more general perspective, the results of this study imply that, to definitively assess the utility of a methodology designed to produce an oriented protein film at a solid−liquid interface, the use of an experimental approach capable of characterizing the molecular orientation distribution in the film is required.

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