Molecular Orientation Distributions in Protein Films. 4. A Multilayer Composed of Yeast Cytochrome *c* Bound through an Intermediate Streptavidin Layer to a Planar Supported Phospholipid Bilayer

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Abstract: Multilayer protein films in which avidin or streptavidin is used as a cross-linking agent are widely used in bioanalytical laboratories for diagnostics, isolation, and localization. However, comparatively little is known about the structure of these films. In this study, molecular orientation in asymmetric protein bilayers assembled on planar glass substrates was investigated. The multilayer architecture consisted of, in order of deposition, a biotin capped phospholipid bilayer, streptavidin, and yeast cytochrome *c* biotinylated at cysteine 102. The orientation distribution of the heme groups in the cytochrome *c* layer was determined using a combination of absorption linear dichroism, measured in a planar integrated optical waveguide-attenuated total reflection geometry, and fluorescence anisotropy, measured in a total internal reflection geometry. A Gaussian model for the orientation distribution was used to recover the mean heme tilt angle and angular distribution about the mean. The orientation distribution for the biotinylated cytochrome *c* layer was 41 ± 11 degrees, which is nearly identical to the orientation distribution measured previously for yeast cytochrome *c* covalently immobilized via cysteine 102 to pyridyl-capped phospholipid bilayers.[12] In that case, the cytochrome surface coverage was 0.9 monolayer. In the present study the cytochrome surface coverage was only 0.4 monolayer, which likely precluded lateral interactions between protein molecules. The fact that the orientation distributions were nearly identical shows that lateral interactions are not required to form a macroscopically oriented protein film.

Introduction

Avidin and streptavidin are tetrameric proteins that are widely used as binding mediators in a diverse array of bioanalytical applications, including chromatography, cytochemistry and cytometry, immunodiagnostics, biosensing, and drug delivery.[1−4] Several features of these proteins have lead to their popularity. First, each contains four binding sites for biotin (vitamin H) and the binding affinity is extremely high ($\log K \approx 15$). Second, under appropriate conditions, the binding interaction is unperturbed when the biotin moiety is covalently attached to a macromolecular carrier or an insoluble substrate. Third, the binding sites are located in pairs on either side of the protein molecule. Thus when avidin or streptavidin is attached to a biotinylated surface or carrier via one or both binding sites on one face of the protein, the other two binding sites on the opposite face are presented to the solution.[5−10] Other biotinylated molecules can then be bound to the free sites. In this manner complex protein film assemblies can be generated for use in diagnostics, isolation, and localization.[3,4] For example, Spinke et al.[7a] fabricated a multilayer assembly on a gold substrate consisting of (*i*) a binary, alkanethiol self-assembled monolayer (SAM) bearing biotinylated tail groups; (*ii*) streptavidin; (*iii*) a Fab fragment biotinylated in the hinge region; (*iv*) the antigen to the Fab fragment, human chorionic gonadotrophin (HCG); and (*v*) an anti-HCG monoclonal antibody. The result was a multilayer assembly designed to biospecifically bind HCG. This type of assembly has been suggested as a model for development of biosensors based on naturally occurring molecular recognition.[8,9]

The extensive use of avidin and streptavidin as binding mediators has fostered considerable interest in relationships between the structure and bioactivity of multilayer films containing these proteins. A significant advance in this field was the discovery that under appropriate conditions both proteins form two-dimensional crystals upon binding to the air/water interface.[10,11] In a pioneering paper,[10] Darst et al. determined

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(1) Chaiet, L.; Wolf, F. *Arch. Biochem. Biophys.* **1964**, *106*, 1−9.
(2) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85−133.
(3) Wilchek, M.; Bayer, E. A.; Eds. Avidin−Biotin Technology. *Methods Enzymol.* **1990**, *184*.
(4) Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988**, *171*, 1−32.
(5) Blankenburg, R.; Meller, P.; Ringsdorf, H.; Salesse, C. *Biochemistry* **1989**, *28*, 8214−8221.
(6) Herron, J. N.; Müller, W.; Paudler, M.; Riegler, H.; Ringsdorf, H.; Suci, P. A. *Langmuir* **1992**, *8*, 1413−1416.
(7) (a) Spinke, J.; Liley, M.; Guder, H.-J.; Angermaier, L.; Knoll, W. *Langmuir* **1993**, *9*, 1821−1825. (b) Schmidt, A.; Spinke, J.; Bayer, T.; Sackmann, E.; Knoll, W. *Biophys. J.* **1992**, *63*, 1185−1192.
(8) Müller, W.; Ringsdorf, H.; Rump, E.; Wildburg, G.; Zhang, X.; Angermaier, L.; Knoll, W.; Liley, M.; Spinke J. *Science* **1993**, *262*, 1706−1708.
(9) (a) Ahlers, M.; Müller, W.; Reichert, A.; Ringsdorf, H.; Venzmer J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1269−1285. (b) Zhao, S.; Reichert, W. M. *Langmuir* **1992**, *8*, 2785−2791.
(10) Darst, S. A.; Ahlers, M.; Meller, P. H.; Kubalek; E. W.; Blankenburg, R.; Ribi, H. O.; Ringsdorf, H.; Kornberg, R. D. *Biophys. J.* **1991**, *59*, 387−396.

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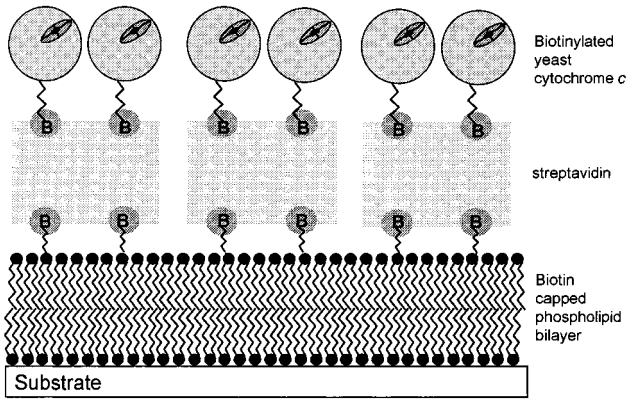

Figure 1. Schematic of the protein bilayer assembly under study. A phospholipid bilayer composed of a 1:20 mol/mol ratio of DOPE-biotin: DOPC is first deposited onto a clean, hydrophilic glass substrate using the Langmuir−Blodgett method. Streptavidin is bound to the biotin groups tethered from the lipid film surface, which leaves two free binding sites facing the solution. Yeast cytochrome *c*, bearing a biotin group linked at Cys102, is bound to the available binding sites on streptavidin.

molecular orientation and packing geometry in a crystalline submonolayer of streptavidin molecules bound to a Langmuir monolayer of biotin-capped lipids. Streptavidin-containing films assembled at solid/liquid interfaces have also been the subject of a number of studies[7−9] but considerably less is known about their structure. These films are usually not crystalline, which makes them incompatible with electron crystallography, the experimental technique employed by Darst et al.[10] (This technique is somewhat limited by the requirements that the protein layer must be crystalline and the measurement must be performed in a vacuum.)

This paper addresses the issue of molecular orientation in a noncrystalline protein film immobilized at a solid/liquid interface using streptavidin as a cross-linker. The control of molecular orientation is clearly an important issue in the development of biomolecular devices based on immobilized protein films, such as biosensors and bioelectronic devices. For example, an antibody immobilized on a solid substrate with the antigenic sites facing the substrate surface will be sterically restricted from binding a macromolecular antigen.[6,7a] Given the extensive use of streptavidin in protein immobilization technologies,[3,4] it is appropriate to examine if binding of a biotinylated protein to a streptavidin-coated substrate produces an oriented protein film. To our knowledge, direct measurements of molecular orientation in noncrystalline protein multilayer films containing streptavidin have not been previously reported.

In a previous paper, we reported that a macroscopically oriented film of yeast cytochrome *c* could be generated using a site-specific immobilization strategy in which the protein is bound via Cys102 to a planar supported phospholipid bilayer bearing pyridyl disulfide (PDS) groups.[12] Here that work is extended to the asymmetric protein bilayer depicted in Figure 1. A biotin-capped phospholipid bilayer is deposited first, followed by a streptavidin layer immobilized via binding to the biotin-capped lipids. The third layer is formed using yeast cytochrome *c* that has been biotinylated at Cys102, which allows it to bind to the free recognition sites on the streptavidin layer. The primary experimental goal of this study was to measure the molecular orientation distribution of the heme groups in the cytochrome (outer) layer of the asymmetric protein bilayer. By comparison to the previous study,[12] we sought to determine if the degree of macroscopic orientation observed for yeast cytochrome *c* directly bonded to a phospholipid bilayer could be maintained through an intermediate layer of biospecifically bound streptavidin. A secondary goal was to measure the orientation distribution of the streptavidin layer.

Experimental Section

Proteins. Streptavidin (Sigma) was dissolved in 50 mM phosphate buffer, pH 7.0, containing 100 mM KCl (referred to hereafter as phosphate buffer). Labeled streptavidin was prepared by reacting the protein with an amine reactive fluorescent probe molecule: either fluorescein isothiocyanate (FITC; Molecular Probes) or anthracene-2,3-dicarboxaldehyde (ADC; Molecular Probes, #A-1139). Streptavidin was modified with FITC using a 1:1 molar ratio of label to protein, as described previously.[5,13] Using absorbance spectrometry, a FITC: streptavidin molar ratio of 0.6 was determined for the labeled product.

A modified procedure based on previous reports[14] was used to label streptavidin with ADC. The protein was dissolved in 50 mM borate buffer, pH 9.5, followed by addition of 300 μL of 10 mM NaCN in 50 mM borate buffer and a molar quantity of ADC equal to the streptavidin. The mixture was allowed to react at room temperature for 30 min before being purified on a Sephadex G-25 column using phosphate buffer as the eluent. An ADC:streptavidin molar ratio of 0.85 was determined for the labeled product using absorbance spectrometry. All streptavidin solutions were passed through 0.4 μm polysulfone membrane filters just prior to preparation of immobilized protein films.

Yeast cytochrome *c* (Sigma) was first purified by cation exchange.[15,16] A 100 μM solution was prepared in 25 mM phosphate buffer containing 10 mM dithiothreitol (DTT) to reduce dimers. The solution was passed through a carboxymethyl cellulose (CM52, Whatman) column, and the protein was eluted with a 25−100 mM ionic strength gradient of sodium phosphate containing 10 mM DTT. Cyt *c* collected from the major band was split into two fractions. One fraction was dialyzed against phosphate buffer for 3 days to remove excess DTT. The second fraction was dialyzed against Type I deionized water for 3 days, followed by evaporation of the water to recover the protein. Zinc-substituted cyt *c* (Zn-cyt *c*), a fluorescent form of the protein, was prepared from the second fraction using procedures outlined previously.[17,18] After purification by size-exclusion chromatography (Sephadex G-25), the Zn-cyt *c* preparation was dialyzed against phosphate buffer.

The thiol reactive reagent *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (Biotin-HPDP, Pierce) was dissolved in a minimal amount of *N*,*N*-dimethylformamide. A 10-fold molar excess of Biotin-HPDP was added to the protein solution (either cyt *c* or Zn-cyt *c*). The reaction was stirred for 3 h at room temperature. After this period, some precipitation of the Biotin-HPDP was observed which was removed by centrifugation. Absorbance measurements were used to quantitate the concentration of the reaction product pyridine-2-thione

(11) (a) Furuno, T.; Sasabe, H. *Biophys. J.* **1993**, *65*, 1714−1717. (b) Ku, A. C.; Darst, S. A.; Kornberg, R. D.; Robertson, C. R.; Gast, A. P. *Langmuir* **1992**, *8*, 2357−2360. (c) Qin, H.; Liu, Z.; Sui, S.-F. *Biophys J.* **1995**, *68*, 2493−2496.

(12) Edmiston, P. L.; Saavedra, S. S., *Biophys J.* **1998**, *74*, in press.

(13) Nargessi, R. D.; Smith, D. S. *Methods Enzymol.* **1986**, *122*, 67−72.

(14) (a) Collins, G. E.; Rose-Pehrsson, S. L. *Analyst* **1994**, *119*, 1907−1913. (b) Kwakman, P. J M.; Koelewijn, H.; Kool, I.; Brinkman, U. A.; DeJong, G. J. *J. Chromatogr.* **1990**, *364*, 155−166. (c) Montigny, P.; Stobaugh, J. F.; Givens, R. S.; Carlson, R. G.; Srinivasachar, K.; Sternson, L. A.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1096−1101.

(15) Brautigan, D. L.; S. Ferguson-Miller, S.; Margoliash, E. *Methods Enzymol.* **1978**, *53*, 128−164.

(16) Willit, J. L.; Bowden, E. F. In *Redox Chemistry and Interfacial Behavior of Biological Molecules*; Dryhurst, G., Niki, K., Eds.; Plenum Press: New York, 1988; pp 63−77.

(17) Robinson, A. B.; Kamen, M. D. In *Structure and Function of Cytochromes*; Okunuki, K., Kamen, M. D., Sekuzu, I., Eds.; University Park Press: Baltimore, 1968; pp 383−387.

(18) Edmiston, P. L.; Lee, J. E.; Cheng, S. S.; Saavedra, S. S. *J. Am. Chem. Soc.* **1997**, *119*, 560−570.

($\epsilon_{343\ nm} = 8080\ M^{-1}\ cm^{-1}$[19]) which is equal to the amount of biotinylated protein. At this point, approximately 25% of the protein was biotinylated (for both cyt *c* and Zn-cyt *c*). Consequently, the procedure was repeated twice more to achieve quantitative biotinylation of protein. The biotinylated proteins (now referred to as B-cyt *c*, and B−Zn-cyt *c*) were purified separately by extensive dialysis for 5 days (two changes per day) against phosphate buffer, followed by chromatography on Sephadex G-25. Immediately prior to preparation of immobilized protein films, protein solutions were passed through 0.4 μm polysulfone membrane filters to remove any particulate matter.

Langmuir−Blodgett Film Deposition. Fused quartz slides (2.5 cm × 7.5 cm × 1 mm thick, Heraeus Amersil) were used as substrates for fluorescence anisotropy measurements. Uranium oxide doped, silica−titania planar waveguides, fabricated by a sol−gel dip coating process[20] were used as substrates for absorbance linear dichroism measurements. All substrates were cleaned by soaking in an 80 °C chromic acid bath for 30 min, rinsing with deionized water (Type I Reagent Grade), light scrubbing with a cotton pad in 0.1% PCC-54 surfactant (Pierce), and again rinsing with deionized water. The substrates were blown dry with N_2 prior to use.

The spreading solution consisting of a 1:20 molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl] (DOPE-biotin, Avanti Polar Lipids) and 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphocholine (DOPC, Sigma 99%) was prepared in chloroform (Aldrich, 99.9%+) at a total lipid concentration of 3 mg/mL. Using this spreading solution, supported phospholipid LB bilayers were deposited on fused quartz and planar waveguide substrates employing the procedure described in a previous paper.[12] To maintain the "head group out" structure of the as-deposited film, LB bilayers were kept under water during all subsequent manipulations.

Protein Film Deposition and Orientation Measurements. The orientation distribution of chromophores in films of immobilized streptavidin and cyt *c* (Figure 1) was examined in separate experiments on separately prepared samples. To prepare an immobilized cyt *c* film for orientation studies, a substrate-supported lipid bilayer was mounted in a flow cell and rinsed with phosphate buffer. A 2 μM solution of unlabeled streptavidin was injected and allowed to bind to the biotin moieties at the bilayer surface. After a 2 h incubation time, the excess streptavidin was rinsed from the cell with phosphate buffer and a 19 μM solution of B-cyt *c* was injected. When preparing samples for fluorescence anisotropy measurements, the solution contained a 1:5 mixture of B−Zn cyt *c*:B-cyt *c*, with a total protein concentration of 19 μM. (B−Zn cyt *c* was diluted with B-cyt *c* to increase the distance between Zn porphyrins in the immobilized cyt *c* layer. This eliminated the possibility of fluorescence energy transfer which could invalidate the anisotropy measurements.[18]) In both cases, the cyt *c* solution was allowed to react with the streptavidin-coated bilayer for 3 h before being flushed from the cell with phosphate buffer. Anisotropy measurements were performed in a total internal reflection fluorescence (TIRF) geometry as described in ref 18. Absorbance linear dichroism measurements were performed using the planar integrated optical waveguide-attenuated total reflection (IOW-ATR) technique, as described previously.[18] Orientation distributions for the porphyrin groups in immobilized cyt *c* films were calculated using a Gaussian distribution model.[18]

To prepare an immobilized streptavidin film for orientation studies, a substrate-supported bilayer was mounted in a flow cell and rinsed with phosphate buffer. The buffer solution was replaced with a 2.0 μM solution of streptavidin in which 10% of the protein molecules were labeled with a fluor, either FITC or ADC. (As described above, the labeled streptavidin was diluted with unlabeled streptavidin to preclude energy transfer in the immobilized protein layer.) After a 2 h incubation time, the protein solution was replaced with phosphate buffer. TIRF anisotropy measurements were performed following the general procedure described previously.[18] The 488 and 514.5 nm lines from an argon ion laser (Ion Laser Technology 5500) were used to excite fluorescence from FITC- and ADC-streptavidin, respectively. Fluorescence was detected through an emission polarizer and a 515 nm long pass filter for FITC-streptavidin films. A 20 nm band-pass filter centered at 568 nm was used in place of the long pass filter for ADC-streptavidin films. Absorbance linear dichroism measurements were not performed on these films for reasons discussed below.

Protein Surface Coverages. Surface coverages for immobilized streptavidin and cyt *c* films were determined using a desorption assay similar to that described in ref 12. Briefly, streptavidin/cyt *c* bilayer protein films were fabricated, as described above, on two 3" × 2" glass microscope slides, each coated on one side. The streptavidin layer was deposited from a 2.0 μM streptavidin solution in which 10% of the protein molecules were labeled with FITC. The cyt *c* layer was deposited from a solution containing a 1:5 mixture of B−Zn cyt *c*:B-cyt *c*, in which the total protein concentration was 19 μM. After forming the multilayer assembly, the protein and LB films were removed by sonication for 10 min in a Teflon vessel containing 12.5 mL of 1% (v/v) Triton X-100 in 50 mM phosphate buffer, pH 7.0, with 200 mM KCl. This procedure completely removed the lipid bilayer and all associated protein as confirmed by epifluorescence microscopy. The solution in the Teflon vessel was transferred to a cuvette, and the fluorescence emission intensity of desorbed FITC-streptavidin and B−Zn-cyt *c* was measured using a Spex Fluorolog fluorometer. FITC-streptavidin fluorescence was excited at 490 nm and emission measured at 520 nm. B−Zn cyt *c* fluorescence was excited at 420 nm and measured at 623 nm. The amount of each protein removed from the substrates was quantified using an external calibration curve. A dilution series containing both FITC-streptavidin and B−Zn-cyt *c* was prepared in a surfactant solution identical in composition to that used to remove the protein and LB films from the slides. An amount of lipid equal in composition and concentration to that composing the desorbed films was added to the calibration solutions. Protein surface coverages were calculated by ratioing the amount of each protein removed to the combined area of the slides, assuming a molecularly flat surface.

Results and Discussion

This study addresses the following question: When a protein is biotinylated at a specific amino acid residue and then adsorbed to a streptavidin-coated planar substrate, is a macroscopically ordered protein film produced? This question is addressed by comparing the molecular orientation distribution of the heme groups in the cyt *c* layer of the multilayer diagrammed in Figure 1 to a previous study[12] in which cyt *c* was directly bonded to a thiol-reactive phospholipid bilayer. Given the widespread use of streptavidin in diagnostic, isolation, and localization techniques,[3,4] the results of this investigation should be of general interest.

A brief description and rationale for the molecular components used to construct the multilayer (Figure 1) follows. A DOPC bilayer is deposited as the base layer because (*i*) it is a relatively inert, biomimetic surface that resists nonspecific protein adsorption;[9a,21] (*ii*) the surface density of the dopant, DOPE-biotin, is easily adjusted; and (*iii*) the chain-melting-phase transition temperature of DOPC is −22° C; thus a bilayer deposited at room temperature is in a single, liquid analogous phase in which the lipids laterally diffuse.[12,22] The latter point is important because lipid fluidity strongly influences binding and packing of streptavidin on a biotin-capped lipid film.[23,24] Binding is inhibited if the lipid film is in a solid-condensed phase.

(19) Stuchbury, T.; Shipton, M.; Norris, R.; Malthouse, J. P. G.; Brocklehurst, K. *Biochem J.* **1975**, *151*, 417−432.

(20) (a) Yang, L.; Saavedra, S. S.; Armstrong, N. R.; Hayes, J. *Anal. Chem.* **1994**, *66*, 1254−1263. (b) Edmiston, P. L.; Saavedra, S. S. *Chem. Mater.* **1997**, 9, 2599−2603.

(21) (a) Tamm, L. K. In *Optical Microscopy: Emerging Methods and Applications*; Herman, B., Lemasters, J. J., Eds.; Academic Press: San Diego, 1993; Chapter 11, and references therein. (b) Sankaram, M. B.; Marsh, D. In *Protein−Lipid Interactions*; Watts, A., Ed.; Elsevier: Amsterdam, 1993; pp 127−162.

(22) Tamm, L. K., McConnell, H. M. *Biophys. J.* **1985**, *47*, 105−113.

As noted above, the extremely high, multidentate affinity of streptavidin for biotin and the symmetry of the binding sites make this protein a very useful cross-linking agent[3,4] and, more recently, a model for studying protein−ligand binding at membrane surfaces.[8,9a] Furthermore, (*i*) streptavidin lacks the polysaccharide groups of avidin which could disrupt the packing of the protein molecules bound to the bilayer, and (*ii*) streptavidin is thought to be less prone to nonspecific adsorption than avidin.[11c,25,26] Yeast cytochrome *c* is used to generate the outermost protein layer because it contains an intrinsic porphyrin group which can be used as a probe of molecular orientation,[12,18] and it contains a single reduced cysteine at position 102[27] which provides a unique site for attachment of a thiol-reactive biotin derivative. Furthermore, molecular orientation measurements on the cyt *c* layer in this multilayer assembly (Figure 1) can be compared to previous results obtained for cyt *c* immobilized directly to a PDS-capped lipid bilayer via disulfide bonding at Cys102.[12]

However, it is important to note that the projected area of a cyt *c* molecule is smaller than the area per biotin binding site on a streptavidin molecule (see below). Given this epitaxial mismatch, a surface coverage significantly less than one monolayer is predicted for biotinylated cyt *c* specifically bound to a streptavidin film. Consequently, the lateral interactions that likely exist between cyt *c* molecules in the near-monolayer film formed on a PDS-capped lipid bilayer[12] must be diminished or absent in a submonolayer film of cyt *c* bound to a streptavidin film. This difference is a significant aspect of this study and is discussed below following presentation of results on the structure, binding properties, and surface coverage of the streptavidin and cyt *c* layers.

Langmuir−Blodgett Film Characterization. The surface pressure versus area isotherms of pure DOPC and 1:20 DOPE-biotin:DOPC were measured (not shown). As expected, these isotherms nearly overlapped since DOPC is the major component in both films and were essentially identical to a previously published isotherm of DOPC (Figure 2 in ref 12). For the 1:20 DOPE-biotin:DOPC film at a surface pressure of 35 mN/m, the area per lipid molecule was approximately 60 Å^2, a typical value for a fluid, packed monolayer.[22,28] Based on the projected area that a streptavidin molecule occupies in a crystalline monolayer (about 3600 Å^2 [10]), the ratio of biotinylated lipids per protein molecule was about 2.9.

To assess the macroscopic uniformity of films formed by LB deposition, bilayers consisting of 1:20 DOPE-biotin:DOPC, doped with a 0.7% molar ratio of the fluorescent lipid 1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3 phosphoethanolamine (NBD-PE, Avanti), were prepared on glass substrates. When examined by epifluorescence microscopy, these films appeared uniformly fluorescent by visual inspection at 400X magnification. This observation was quantitatively confirmed by TIRF measurements of emission intensity at several locations on the surface of each slide. Film uniformity was unaffected by storage under water overnight or by manipulations such as rinsing with buffer solutions.

Epifluorescence of Protein Films. To access the macroscopic quality of streptavidin films, FITC-streptavidin was allowed to bind to 1:20 DOPE-biotin:DOPC phospholipid bilayers which were subsequently observed by epifluorescence microscopy. A uniform fluorescence emission was observed which lacked any observable structural features at 400X magnification. Bilayer protein films consisting of unlabeled streptavidin and 1:5 B−Zn-cyt *c*:B-cyt *c* were also observed by epifluorescence. Although exhibiting only a faint fluorescence, these films also appeared macroscopically uniform with an absence of structural features. The uniformity was confirmed by TIRF measurements. The relative standard deviation in B−Zn-cyt *c* emission intensity measured at 10 spots on two separate films was 6%.

Streptavidin Orientation Distribution. Unlike cytochrome *c*, streptavidin lacks an intrinsic spectroscopic probe that can be used to perform polarized spectroscopic experiments in the visible spectrum. However, from prior work[5] it appears that streptavidin may be preferentially labeled at a single lysine residue under conditions where the labeling ratio is equimolar. This suggests that a structurally unique site on the protein can be labeled with a fluorescent tag that may be used as a probe of molecular orientation. FITC-streptavidin was consequently prepared and immobilized on lipid bilayers doped with DOPE-biotin. TIRF measurements on these films yielded an anisotropy of -0.172 ± 0.032 ($n = 3$) for the FITC probe. The theoretical anisotropy that would be measured in a TIRF geometry for a completely isotropic distribution of linear dipoles is -0.2.[29] Thus the experimental result meant that (*i*) the streptavidin layer immobilized on the lipid bilayer is relatively disordered, or (*ii*) probe labeling occurred at multiple sites, rather a single site, on the surface of the protein, or (*iii*) significant rotational motion of the FITC probe occurs during the lifetime of the excited state. Any of these situations would produce depolarized fluorescence emission, but since FITC is bound to streptavidin through a single bond, the latter situation appeared most probable.

To test the latter hypothesis, intrinsic fluorescence anisotropy (r_o) measurements were performed.[18] For FITC-streptavidin dissolved in 30% (v/v) glycerol in phosphate buffer, r_o was 0.306 from which γ, the angle between the absorption and emission dipoles, was calculated to be 23° using eq 9 in ref 18. For FITC immobilized in a cross-linked silicone rubber (RTV 615, General Electric), r_o was 0.394 and $\gamma = 6°$. Approximating the streptavidin molecule as a sphere, a rotational correlation time of 80 ns was calculated for the protein dissolved in 30% glycerol.[30] Since 80 ns is considerably longer than the radiative lifetime of fluorescein (about 4.5 ns[31]), significant depolarization of fluorescence emission from FITC-streptavidin could not be caused by global motion of the protein molecule and thus can only be ascribed to localized motion of the fluor. In other words, the differences in measured r_o and calculated γ values for FITC-streptavidin and FITC established that significant rotational motion of the probe was occurring despite the fact that it was covalently linked to the protein.

The results obtained with FITC prompted additional experiments using a second fluorescent labeling reagent. ADC forms a five-membered ring with the amino terminus of a lysine residue,[14] which should prevent free rotation about the label-lysine bond. However, the anisotropy of ADC-streptavidin

(23) Weisenhorn, A. L.; Schmitt, F.-J.; Knoll, W.; Hansma, P. K. *Ultramicroscopy* **1992**, *42*−*44*, 1125−1132.
(24) Reiter, R.; Motschmann, H.; Knoll, W. *Langmuir* **1993**, *9*, 2430−2435.
(25) Duhamel, R. C.; Johnson, D. A. *Methods. Enzymol.* **1990**, *184*, 201−207.
(26) Green, N. M. *Methods Enzymol.* **1990**, *184*, 51−67.
(27) Brayer, G. D.; Murphy, M. E. P. In *Cytochrome c: A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science: Sausalito, CA, 1996; Chapter 3.
(28) Yue, B. Y.; Jackson, C. M.; Taylor, J. A. G.; Minigins, J.; Pethica, B. A. *J. Chem. Soc., Faraday Trans.* **1976**, *72*, 2685.
(29) Edmiston, P. L.; Lee, J. E.; Wood, L. L.; Saavedra, S. S. *J. Phys. Chem.* **1996**, *100*, 775−784.
(30) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983; p 143.
(31) (a) Chen, R. F.; Vurek, G. G.; Alexander, N. *Science* **1967**, *156*, 949. (b) Ware, W. R.; Baldwin, B. A. *J. Chem. Phys.* **1964**, *40*, 1703.

Table 1. Orientation Distributions Calculated for Selected Combinations of Emission Anisotropy and Dichroic Ratio: Application to B-Cyt *c* Bound to Streptavidin Immobilized on a Biotin-Capped Phospholipid Bilayer[a]

	anisotropy (r)		
dichroic ratio (ρ)	-0.096	-0.107 ± 0.011[b]	-0.118
1.12	$40° \pm 14°$	*n/o*[c]	*n/o*
1.16	$40° \pm 9°$	$42° \pm 14°$	$44° \pm 21°$
1.18 ± 0.057[b]	$40° \pm 7°$	$41° \pm 11°$	$43° \pm 17°$
1.20	*n/o*	$41° \pm 9°$	$42° \pm 14°$
1.24	*n/o*	$41° \pm 7°$	$42° \pm 11°$

[a] Gaussian orientation distributions expressed as $\theta_\mu \pm \theta_\sigma$. [b] Measured ρ and r values (mean ± standard deviation) for B-cyt *c* bound to streptavidin immobilized to biotin capped phospholipid bilayers. [c] Not obtainable. The pair of values is physically inconsistent with a Gaussian orientation distribution of circularly polarized oscillators.

immobilized on lipid bilayers doped with DOPE-biotin was -0.175 ± 0.053 ($n = 3$), nearly identical to the results obtained with FITC-streptavidin. Intrinsic fluorescence anisotropy measurements confirmed that significant free rotation of the label was occurring, presumably about the single bonds in the lysine side chain. Thus despite the difference in chemical structure, the use of ADC did not prevent depolarization due to local rotational motion.

Steady-state anisotropy cannot be used as a measure of molecular orientation if significant rotational motion of the fluor occurs during its radiative lifetime. Consequently, absorbance linear dichroism measurements were not performed on immobilized FITC- or ADC-streptavidin films, and efforts to determine the orientation distribution of these samples were discontinued.

Cytochrome *c* Orientation Distribution. The heme group of cyt *c* is bound to the polypeptide via two thioether bonds and two axial ligands to the Fe atom.[27] Thus this chromophore should not be subject to the problems associated with local probe motion in labeled streptavidin.

Three multilayer films, having the structure illustrated schematically in Figure 1, were prepared on both planar waveguide and fused quartz substrates for IOW-ATR linear dichroism and TIRF anisotropy measurements, respectively. The dichroic ratio (ρ) of the porphyrin groups was 1.18 ± 0.06 ($n = 3$), while the anisotropy (r) was -0.107 ± 0.011 ($n = 3$). Using the mean ρ and r values, the orientation distribution of porphyrin tilt angles ($\theta_\mu \pm \theta_\sigma$) was calculated to be $41° \pm 11°$, assuming a Gaussian distribution function. Within one standard deviation of the mean ρ and r values, other theoretically consistent combinations of θ_μ and θ_σ could also be calculated. However, as shown in Table 1, there was little variation in the calculated orientation distribution within this range. Specifically, within ± 0.06 for ρ and ± 0.011 for r, θ_μ varied from 40° to 44° while θ_σ varied from 7° to 21°. The orientation distribution of $41° \pm 11°$ is therefore considered representative of the distributions that could be calculated for combinations of ρ and r within one standard deviation of their mean values.

This result can be compared to a previous study[12] in which cyt *c* was immobilized directly to a phospholipid bilayer, primarily via disulfide bonding between Cys102 and lipids bearing PDS moieties. In that case, the measured ρ and r values were 1.13 ± 0.10 and -0.101 ± 0.012, respectively, yielding a porphyrin orientation distribution of $40° \pm 11°$. These parameters are nearly identical to those given above for the asymmetric bilayer. Thus macroscopic molecular orientation in the cyt *c* layer is unchanged despite the presence of the intermediate streptavidin layer. The similarity is graphically displayed in Figure 2 where the normalized Gaussian orientation distributions for both molecular architectures are plotted.

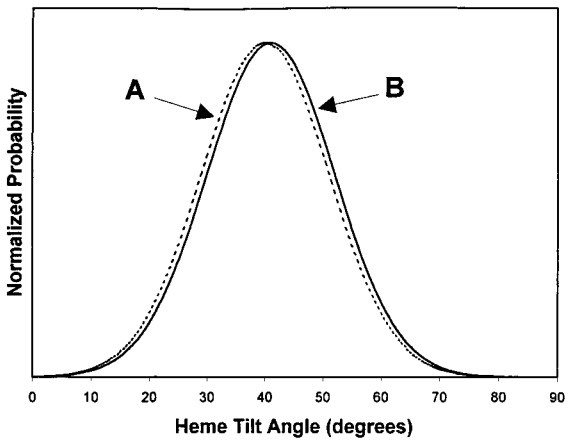

Figure 2. Gaussian probability distributions for (A) (dotted line) yeast cyt *c* immobilized on a pyridyl disulfide-capped phospholipid bilayer, $\theta_\mu = 40°$ and $\theta_\sigma = 11°$, from ref 12 and (B) (solid line) biotinylated yeast cyt *c* bound to streptavidin immobilized to a biotin-capped phospholipid bilayer, $\theta_\mu = 41°$ and $\theta_\sigma = 11°$, this study.

Binding Assays. Binding assays were performed to investigate if the protein layers in the asymmetric bilayer were biospecifically immobilized as depicted in Figure 1. The first series of measurements was designed to determine if streptavidin was biospecifically bound to biotin groups at the surface of the lipid bilayer. Two sets of planar lipid bilayers were prepared. One set was composed of 1:20 DOPE-biotin:DOPC; the other set was composed of pure DOPC which functioned as the control. FITC-streptavidin was incubated with each set of films. After 2 h the samples were rinsed with phosphate buffer, without exposure to air, and the FITC emission intensity was measured using epifluorescence microscopy to determine the relative amount of protein bound to each type of film. The emission intensity from samples formed on bilayers containing DOPE-biotin was nearly eight times greater than that from samples formed on pure DOPC bilayers ($n = 2$). Thus the majority of the streptavidin immobilized on DOPE-biotin:DOPC bilayers was biospecifically bound rather than nonspecifically adsorbed.

A second series of experiments was designed to determine if B-cyt *c* was biospecifically bound to the immobilized streptavidin layer. Supported bilayers consisting of 1:20 DOPE-biotin: DOPC were prepared on quartz substrates followed by adsorption of streptavidin. After rinsing in phosphate buffer, the samples were divided into two sets. One set was incubated with 100 μM biotin dissolved in phosphate buffer followed by a buffer rinse; this treatment was designed to saturate the free binding sites on the immobilized streptavidin layer. Both sets of films were then incubated for 3 h in a 1:5 B−Zn-cyt *c*:B-cyt *c* solution in which the total protein concentration was 19 μM. After the cyt *c* solution was replaced with phosphate buffer, the emission intensity of the bound B−Zn-cyt *c* was measured using epifluorescence microscopy to determine the relative amount of protein bound to each film. The emission intensity of films that had not been blocked with soluble biotin was about 8-fold greater than the intensity emitted from blocked (control) films ($n = 2$). This result shows that the binding of B−Zn-cyt *c* to immobilized streptavidin was predominately biospecific in nature.

Desorption Assays. Biotin-HPDP reacts with a thiol to generate a disulfide bond.[32] Treatment of the asymmetric protein bilayer depicted in Figure 1 with DTT should therefore liberate biospecifically bound B-cyt *c*. Consequently, a series

(32) Biotin-HPDP (21341). Technical bulletin; available from Pierce, Rockford, IL.

Table 2. Desorption of B-Cyt *c* from Streptavidin Immobilized on a Biotin-Capped Phospholipid Bilayer by Salt and Dithiothreitol Solutions

desorption treatment	%age of initial fluorescence intensity remaining after applying desorption treatments to B-Cyt *c* films[a]
buffer rinse[b]	100%
buffer containing 200 mM KCl, 15 min static incubation	93% ± 12% (n=3)
buffer containing 200 mM KCl, 8 h static incubation	91% ± 7% (n = 3)
buffer containing 200 mM KCl, 10 mM DTT, 15 min static incubation	52% ± 8% (n = 3)
buffer containing 200 mM KCl, 10 mM DTT, 8 h static incubation	35% ± 9% (n = 3)

[a] Protein films were formed from solutions containing 1:5 B−Zn cyt *c*:B-cyt *c*, under the same conditions used to form films for TIRF anisotropy measurements (see 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 saline buffer. [b] 50 mM phosphate buffer, pH 7.0, containing 100 mM KCl.

of desorption experiments was performed to determine what fraction of the B-cyt *c* layer was actually immobilized to the streptavidin layer via the biotin-HPDP linker. The general procedure has been described previously.[12,18,33] Briefly, three protein bilayer films were prepared using the same procedure employed to prepare films for TIRF anisotropy measurements of immobilized B−Zn-cyt *c*. Epifluorescence microscopy was used to quantitatively monitor the extent of Zn-cyt *c* desorption effected by soaking the films in buffer solutions containing a high salt concentration, DTT, or both.

The results are listed in Table 2. After the films were soaked for 15 min in phosphate buffer containing 200 mM KCl (ionic strength of 313 mM), 93% ± 12% of the B−Zn-cyt *c* layer that was initially immobilized remained bound. No additional desorption (91% ± 7%) was observed by extending the soaking period to 8 h. When these films were subsequently soaked in phosphate buffer containing 200 mM KCl and 10 mM DTT for 15 min, the relative amount of B−Zn-cyt *c* remaining bound dropped to 52% ± 8%. Extending the treatment with DTT to 8 h left only 35% ± 9% of the initial B−Zn-cyt *c* layer bound to the films. These results are summarized as 5%−10% of the immobilized B-cyt *c* layer is electrostatically adsorbed, approximately 60% is specifically bound via the biotin linker, and the remaining 35% is bound by unknown interactions that cannot be disrupted by the combination of DTT and high salt. These results correlate reasonably well with the results of the binding assays described above. Furthermore, they are remarkably similar to the results of analogous desorption experiments performed on yeast cyt *c* immobilized directly to a PDS-capped lipid bilayer.[12] In those films, 5%−10% of the cyt *c* layer was electrostatically adsorbed, approximately 60% was immobilized via disulfide bonding, and about 35% could not be desorbed in a buffer containing KCl and DTT.

Protein Surface Coverages. The protein bilayer was further characterized by measuring the surface coverage of each protein. The film was found to be composed of 2.6×10^{-12} mol/cm^2 of streptavidin and 8.8×10^{-12} mol/cm^2 of B-cyt *c*. These values can be expressed as monolayer fractions by assuming a packing density for each protein layer. On a molecularly flat surface, a close packed cyt *c* monolayer corresponds to a packing density of 2.2×10^{-11}mol/cm^2,[18] from which a fractional coverage of 0.4 monolayer is calculated for B-cyt *c*.

The packing density reported by Darst et al.[10] is assumed for the streptavidin layer (despite the difference in film fabrication methods). In their work, streptavidin was bound to a biotinylated lipid monolayer at the surface of a Langmuir trough, followed by compression and transfer to an electron microscope grid. The protein formed crystalline domains with a unit cell dimension of 84 ± 1 Å × 85 ± 2 Å,[10] which corresponds to a monolayer surface coverage of 4.65×10^{-12} mol/cm^2. The surface coverage measured here, 2.6×10^{-12} mol/cm^2, therefore represents a fractional coverage of 0.6 monolayer. A submonolayer coverage was not unexpected; other groups studying streptavidin binding to planar biotinylated surfaces have also reported data consistent with submonolayer coverage.[7]

The surface coverage measurements show that the molar ratio of B-cyt *c* to streptavidin in the bilayer was 3.4. Since there cannot be more than two B-cyt *c* binding sites per immobilized streptavidin molecule, these data show that a significant fraction (approximately 40%) of the molecules in the B-cyt *c* layer *were not* biospecifically immobilized. This result correlates well with the desorption data which indicated that about one-third of the B-cyt *c* layer was nonspecifically adsorbed. Some nonspecific adsorption of cyt *c* was expected. This statement is based on two points. (*i*) In the previous study[12] in which yeast cyt *c* was immobilized directly to a PDS-capped lipid bilayer composed predominately of DOPC, some nonspecific protein adsorption to the bilayer surface was observed. (*ii*) Since the streptavidin surface coverage was only 0.6 monolayer, a considerable fraction of the lipid bilayer surface was available for interaction with dissolved cyt *c* molecules.

Macroscopic Orientation in the Cytochrome *c* Layer − Contributing Factors. One key result of this study is that the orientation distribution of the porphyrin groups in a film of B-cyt *c* immobilized through an intermediate streptavidin layer (Figure 1) is nearly identical to that measured previously[12] for a film of cyt *c* covalently immobilized to a PDS-capped phospholipid bilayer. In both molecular architectures, the majority of the cyt *c* (approximately 60%) is site-specifically bound and the width of the measured distribution (± 11°) is relatively narrow, which correlates with a predominantly site-specific interaction. An earlier study[33] showed that a much broader distribution, corresponding to the deposition of a relatively disordered protein film, occurs when extensive nonspecific binding interferes with the intended site-specific interaction.

On the other hand, the desorption and surface coverage measurements show that about 35−40% of the B-cyt *c* layer is nonspecifically bound. This result appears to be inconsistent with the measured orientation distribution (41 ± 11 degrees), which indicates that the porphyrin groups in the cyt *c* layer are macroscopically oriented. However, a similar degree of nonspecific binding was observed in the previous study[12] for cyt *c* deposited directly on a PDS-capped lipid bilayer. In that case, selective desorption of the specifically bound fraction was used to measure the orientation distribution of the nonspecifically bound fraction (33 ± 40 degrees), which allowed the distribution of the specifically bound fraction to be recovered (39 ± 9 degrees). Since the means of the two subpopulations were not significantly different, the orientation distribution of the entire film (40 ± 11 degrees) was not significantly broadened. A similar situation may have occurred for the bilayer geometry examined here. However, this possibility was not further investigated because the surface coverage of the nonspecifically

(33) Wood, L. L.; Cheng, S.-S.; Edmiston, P. L.; Saavedra, S. S. *J. Am. Chem. Soc.* **1997**, *119*, 571−576.

bound B-cyt *c* (about 0.14 monolayer) was too low to perform statistically valid linear dichroism measurements.

Although the orientation distribution of the streptavidin layer of the protein bilayer was not measured, macroscopic order in this layer is one probable reason that the orientation distribution of the cyt *c* layer is nearly identical to that measured for the PDS-capped lipid bilayer geometry.[12] As noted above, under appropriate conditions streptavidin forms crystalline domains at the air−water interface in which the biotin binding sites are oriented approximately normal to the interfacial plane.[5,10,11] The streptavidin films prepared in this study were probably not crystalline since characteristic morphologies were not observed when FITC-streptavidin films were examined by epifluorescence microscopy. However, given the nearly identical orientation distributions plotted in Figure 2, the streptavidin layer must have a high degree of macroscopic order that is "transferable" to the subsequent cyt *c* layer.[8]

A second key result of this study is that the fractional surface coverage of the cyt *c* layer was 0.4 monolayer, which is less than half of the 0.9 monolayer coverage achieved when cyt *c* was immobilized directly to a PDS-capped lipid bilayer.[12] Considering this difference in light of the nearly identical orientation distributions measured for these two architectures leads to a significant conclusion: lateral protein−protein interactions are *not* a prerequisite for the generation of an macroscopically oriented cyt *c* film. B-cyt *c* molecules bound to different streptavidin molecules in the packing arrangement reported by Darst et al.[10] would be too widely spaced to interact laterally (although a pair of cyt *c* molecules bound to one streptavidin might be close enough). Even if the streptavidin molecules formed a close-packed structure, in which the projected area per molecule would be about 2500 Å^2,[34] the area per biotin binding site (1250 Å^2) would still be considerably larger than the area of a cyt *c* molecule ($\leq$900 Å^2). Therefore it appears that, at least in this molecular architecture, specific binding of B-cyt *c* to streptavidin determines porphyrin orientation, and lateral interactions are relatively unimportant. In contrast, other authors have suggested that lateral interactions among proteins occupying adjacent adsorption sites may be necessary to "force" the molecules to align in a particular geometric orientation, possibly by sterically hindering translational and rotational motion and/or preventing multiple types of adsorptive interactions with the adsorbent surface.[35]

The length of linker between B-cyt *c* and streptavidin is also likely to be a significant factor in generating a narrow distribution of porphyrin tilt angles. The distance from the reactive disulfide group to the biotin ring structure in Biotin-HPDP is 29.2 Å.[32] From an examination of the crystal structures of the two proteins[36] we estimate the following: (*i*) The distance from a biotin group occupying a binding site on streptavidin to the outer surface of the protein is 17 Å. (Alternately, from the diagram published by Darst et al. [Figure 8 in ref 10], this distance is estimated to be 15 Å). (*ii*) The thiol side chain of Cys102 is located 8 Å from the surface of cyt *c* (although partial unfolding of the C-terminal region to permit reaction with an extrinsic thiol could conceivably shorten this distance[27]). Given these distances, only about 5 Å of the spacer arm is left to span the "gap" when B-cyt *c* is bound to streptavidin. The resulting steric hindrance probably restricts the motion of bound B-cyt *c*, which in turn should restrict the porphyrin to a narrow range of tilt angles. Finally, electrostatics may also play a role. At pH 7.0, cyt *c* has a net charge of +9, whereas, with a pI between 5 and 6,[26] streptavidin has a net negative charge. Therefore the binding interaction between the proteins may be enhanced by electrostatic attraction.

Conclusion

Due to their extremely high, multidentate affinity for biotin, avidin, and streptavidin have become widely used as binding mediators in a diverse array of bioanalytical applications.[3,4] In contrast to this popularity, relatively little is known about the structure of protein multilayers formed using either avidin or streptavidin as a cross-linking agent. In this work, the issue of molecular orientation in the outermost layer of a streptavidin-mediated protein multilayer was addressed. Multilayer assemblies consisting of B-cyt *c* bound to a biotin capped phospholipid bilayer through an intermediate streptavidin layer were structurally characterized. The orientation distribution of the porphyrin groups in the B-cyt *c* layer was almost identical to the distribution in films formed by direct immobilization of cyt *c* to PDS-capped lipid bilayers.[12] Thus the intermediate streptavidin layer did not measurably affect macroscopic molecular orientation in the outer B-cyt *c* layer. The low surface coverage of the B-cyt *c* layer, ca. 0.4 monolayer, demonstrates that formation of a close packed monolayer on a streptavidin-coated surface is *not required* to generate an oriented protein film.

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(34) A streptavidin molecule bound to a biotinylated surface occupies an area of approximately 45 Å × 55 Å.[10]

(35) See, for example: (a) Morrissey, B. W. *Ann. N. Y. Acad. Sci.* **1977**, *28*, 50−64. (b) Jönsson, U.; Lundström, I.; Rönnberg, I. *J. Colloid Interface Sci.* **1987**, *117*, 127−138.

(36) Crystal structures were obtained from the Brookhaven data bank and viewed using the INSIGHT II software package (Biosym Technologies, San Diego, CA).