were further minimized through molecular mechanics using the MM2 program in a Tektronik CAChe workstation. The energies given in the tables are calculated from a vacuum and are to be considered as qualitative only to support the experimental observation.

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Genetic Engineering of Surface Attachment Sites Yields Oriented Protein Monolayers†

Patrick S. Stayton, Jill M. Olinger, Min Jiang, Paul W. Bohn,* and Stephen G. Sligar*

Contribution from The Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, 405 North Mathews, Urbana, Illinois 61801.
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Abstract: There has been considerable interest in the use of self-assembly and Langmuir-Blodgett techniques to generate ordered macromolecular monolayers. We describe a general method of using genetic engineering to produce a unique chemical group on the surface of a protein at a predefined site which can be used to orient the macromolecule in a self-assembled film. Using the heme protein cytochrome b_5, produced from a totally synthetic gene, allows direct determination of orientation through measurement of absorbance linear dichroism.

There is considerable current interest in the use of biologically derived macromolecules for materials applications.[1] However, while the naturally occurring variety of protein structure and function is impressive, biomaterial fabrication is inherently limited by the availability and inflexibility of the native protein pool. Protein engineering techniques offer, in principle, the means to circumvent these limitations by allowing the equivalent of synthetic flexibility. For example, genetic engineering techniques represent a potential means to achieve the defined assembly of protein thin films. The construction of close-packed monolayers of oriented cytochrome b_5 on optical substrates has been achieved through the introduction of unique thiol functionalities at defined surface sites. These protein thin films are self-assembled through the covalent linkage of cysteine mutants with a sulfhydryl specific silane layer, and linear dichroism measurements of the resultant heme orientation show that non-random monolayer assemblies are formed. Most importantly, differential heme orientation can be achieved through the choice of attachment site, demonstrating a striking means to manipulate molecular orientation in protein monolayers.

Cytochrome b_5 is a small, 13 000 daltons, bis-imidazole-ligated heme protein for which high resolution crystal and solution structures are available.[2,3] Heme proteins are attractive candidates for biomaterial fabrication due to the unique optical and electronic properties associated with the heme prosthetic group.[4] Because the heme orientation is constrained by the protein scaffolding, orientation of the prosthetic group relative to the substrate should be controlled by the stereochemistry of the protein-surface interactions. We reasoned that a first-order manipulation of protein-surface interactions could be accomplished by genetically engineering unique attachment sites on the cytochrome b_5 surface for covalent linkage with a derivatized surface.

Site-directed mutagenesis techniques were utilized to produce two mutants by introducing a unique thiol functional group on the protein surface at two independent points, replacing threonine 8 (mutant T8C) and, separately, threonine 65 (mutant T65C).[5] Glass substrates were prepared by treatment with a silane coupling agent, (3-iodopropyl)trimethoxysilane.[6] Surface coverage measurements utilized the pyridine hemochrome assay for measuring the heme content on derivatized slides.[7] Typical measurements yielded ca. $1.1-1.4 \times 10^{13}$ molecules/cm^2, corresponding to 700-900 Å^2 per protein molecule. The crystallographic dimensions of cytochrome b_5 are approximately $30 \times 25 \times 25$ Å, which suggests that nearly complete monolayer coverage has been achieved. Covalent attachment of cytochrome b_5 to the surface through specific thioether linkages was verified by extensive buffer washings to remove physisorbed protein and comparisons to control experiments with wild-type b_5.

A low-noise single-pass linear dichroism method was utilized to provide the necessary sensitivity for measuring the heme orientation in cytochrome b_5 monolayer assemblies.[8] Because the

* Authors to whom correspondence should be addressed.

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(6) Ti:Zn glass microscope cover slips (60 × 24 mm, Corning) were acid/base washed (3 hot H_2SO_4 × 3 min/hot $NH_4OH-H_2O_2$ (4:1) × 3 min) and derivatized by refluxing 1% solutions of (3-iodopropyl)trimethoxysilane (Petrarch) in dried chloroform (3 h). Siloxane coverage was estimated by spectrophotometric titration of (3-mercaptopropyl)trimethoxysilane treated substrates with dithionitrobenzoic acid and contact angle measurements. Free thiol quantitation yielded ca. 64 A^2/SH group, and water contact angles of 55-60° were measured. Protein modification was executed in Teflon tanks allowing for submersion of half the slide in 50 mM phosphate buffer, pH 7.5-8.0, 5 mM EDTA, with overnight incubation followed by consecutive buffer and distilled H_2O rinses, and final drying under N_2. Control pyridine hemochrome assays and linear dichroism measurements were conducted on slides exposed to wild-type cytochrome b_5 and buffer alone. No heme was measureable and no linear dichroism relative to the blank was observed in either control.

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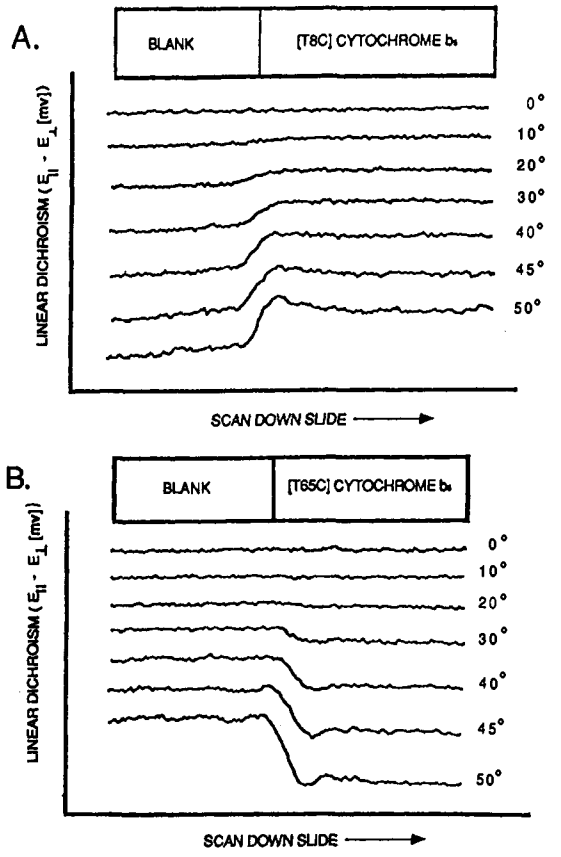

Figure 1. Linear dichroism of oriented protein monolayers. Translational scans of linear dichroism at 410 nm down the long axis of derivatized cover-slips. The lock-in amplifier detected ac signal is plotted on the *y* axis for seven tilt angles (defined relative to 0 at the normal to the direction of light propagation). The discrete transition from blank to protein sides is due to the presence of a meniscus at the air–water interface of the protein derivitization tank. The measurements of linear dichroism for the T8C mutant (A) and for the T65C mutant (B) were conducted under identical signal and reference phase relationships.

absorption transition moment at 410 nm lies in the porphyrin plane,[9] the linear dichroism measured is proportional to the tilt angle of the heme plane relative to the glass substrate. The sample is fabricated such that only half is coated with cytochrome b_5, allowing for the subtraction of intrinsic contributions to the optical dichroism signal on the blank side (primarily Fresnel reflection).

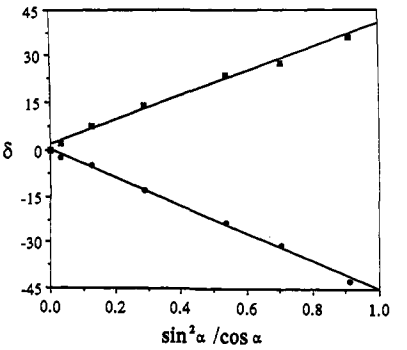

Figure 2. Differential orientation of protein assemblies directed by sulfhydryl attachment site. A plot (lines represent linear regression fits) of the linear dichroism normalized to the heme absorption [δ = ac (lock-in signal)/dc] vs the theoretical geometric factor for the planar heme absorption transition moment, $\sin^2\alpha$.[8a] The sign of linear dichroism is invariant for T8C (■) and T65C (●) from preparation to preparation, with only small variations in the magnitudes of δ.

Measurements of the differential absorption of linearly polarized light were plotted across the slide from the blank to coated regions as a function of the slide tilt angle, α (Figure 1).

Three important observations can be derived from these plots. First, the α-dependent differences in orthogonal polarized light absorption clearly demonstrate that a non-random orientation of heme prosthetic groups exists in the cyltochrome b_5 monolayer assemblies, as a random distribution (corresponding to an observed angle of 54.7°) would yield a linear plot with zero dichroism at all slide tilt angles. Second, the excellent coverage homogeneity of the cytochrome b_5 orientation is evident in the translational linearity of the dichroism effect across the protein coated portion of the thin film. Finally, and most striking, is the control over heme orientation provided by the choice of the genetically engineered surface attachment site (Figure 2). The T8C and T65C cytochrome b_5 assemblies exhibit very distinct linear dichroism effects, with opposite relative signs for the optical signal representing parallel minus perpendicular light component absorption. The linear dependence of the dichroic ratio on the geometric factor $\sin^2\alpha/\cos\alpha$ is as predicted for the linear absorption transition of the heme prosthetic group.[8a] The change in sign for the two mutants specifies that the average tilt angles for the two mutants lie on either side of 54.7°.[8a] We are currently conducting experiments aimed at more precisely quantitating the average tilt angle (which is complicated by the degeneracy of the linear absorption transition in the heme plane) and also at determining the distribution of tilt angles around the mean. These comparisons are made under similar close-packing concentrations and do not simply reflect variations in orientation distribution arising from differential conformational freedom. We are currently extending attempts to control heme orientation through the use of double attachment sites, the construction of substrates with controlled surface properties, and the engineering of protein surface electrostatic potentials to control protein–protein and protein–surface interactions.[10]

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