Orientation of adsorbed cytochrome *c* as a function of the electrical potential of the interface studied by total internal reflection fluorescence

J. G. E. M. Fraaije,* J. M. Kleijn, M. van der Graaf, and J. C. Dijt
Department of Physical and Colloid Chemistry, Wageningen Agricultural University, 6703 HB Wageningen, The Netherlands

ABSTRACT A method for determination of the orientation of adsorbed structure-stable proteins using Total Internal Reflection Fluorescence is outlined. The theory has been elaborated for orientation studies on adsorbed free base cytochrome *c*, of which the prophyrin can be used as an intrinsic fluorescent label. The ratio of fluorescence intensities at two polarization modes of the incident light (the transverse magnetic and the transverse electric polarization mode, respectively) gives a relation between the orientation angles of the porphyrin relative to the interface. As an illustration of the theory, experimental results on the adsorption of cytochrome *c* at an optically transparent SnO_2 film electrode are presented. It is concluded that the orientation of the molecules can only be affected by the interfacial potential during the process of adsorption, but, once adsorbed, the orientation cannot be changed anymore by variation of the potential.

INTRODUCTION

Total internal reflection fluorescence (TIRF) is a very promising spectroscopic technique to study protein adsorption in situ. It can be used to follow adsorption and desorption kinetics and qualitative information on conformational changes in adsorbing proteins can be obtained. Reviews of the various aspects of the TIRF approach to protein adsorption are given in references 1–4.

This paper discusses a new application of TIRF, namely the quantitative determination of the orientation of adsorbed protein molecules. Basic prerequisites are that the protein of interest has a fluorescent group with a well-known and constant orientation relative to the rest of the molecule and that it does not change its conformation upon adsorption. Emphasis is put on principles and methods, especially elaborated for adsorption of the free-base derivate of cytochrome *c*. This is illustrated by results of first experiments. TIRF orientation measurements were performed at the surface of an optically transparent electrode (OTE). An OTE consists of a very thin film of (semi)conducting material on an optical transparent substrate. This extension of the technique offers the possibility to study the orientation of the adsorbed protein as a function of the electrical potential of the interface.

The principle to use TIRF for orientation measurements has been applied before by Thompson et al. (5, 6) to characterize the order in phospholipid monolayers. OTE's have been used already for ~25 yr to follow electrochemical reactions with spectroscopic techniques (see e.g., references 7–9), but, to our knowledge, they have not been applied before to study the orienting effect of the electrical potential of the interface on adsorbing molecules.

THEORY

General

TIRF theory has been outlined before (3, 4, 10) and therefore only the principle is mentioned here. A light beam striking an interface between two media 1 and 2, with refractive indices n_1 and n_2, is totally reflected if $n_1 > n_2$ and the angle of incidence $\theta_i > \sin^{-1}(n_2/n_1)$. At the point of total reflection an evanescent wave penetrates into medium 2. The amplitude of this stationary electromagnetic wave decays exponentially with distance normal to the surface. When medium 2 consists of a solution of fluorescent molecules that absorb at the wavelength of the incident light, the molecules in the evanescent field will fluoresce. In the case of adsorption and not too high bulk concentrations, most of the fluorescence intensity will be due to molecules in the adsorption layer.

Determination of the orientation of adsorbed molecules

The fluorescence intensity of a molecule depends in a linear way on the absorption intensity. The absorption

*Dr. Fraaije's present address is AKZO Research Laboratories Arnhem, P.O. Box 60, 6800 AB Arnhem, The Netherlands.

Address correspondence to Dr. Kleijn at Department of Physical and Colloid Chemistry, Wageningen Agricultural University, Dreijenplein 6, 6703 HB Wageningen, The Netherlands.

intensity A is given by:

$$A = \langle(\mu \cdot \mathbf{E})^2\rangle, \quad (1)$$

where μ is the transition dipole moment of the molecule and $\mathbf{E}$ the electric field component of the evanescent field; $\langle\ \rangle$ denotes a time average. From Eq. 1 it is clear that the absorption intensity, and therefore the fluorescence intensity, depends on the angle between the transition dipole moment of the chromophore and the electric field vector. The relative magnitudes of the components E_x, E_y, and E_z of $\mathbf{E}$ depend on the polarization angle Ψ of the incident light, the refractive indices of the media, and the angle of incidence θ_i(10, 11). Using different polarizations of the incident light, it is possible to obtain information about the orientation or orientation distribution of the adsorbed molecules.

In the case of free base cytochrome *c*, the porphyrin ring has two transition dipole moments with interchangeable positions. The situation of one transition dipole per molecule has been analyzed by Thompson et al. (5). For simple reference, the coordinate system used by these authors is given in Fig. 1 *a*. The transition dipole moment is expressed as

$$\mu = |\mu| \begin{pmatrix} \sin\theta\cos\phi \\ \sin\theta\sin\phi \\ \cos\theta \end{pmatrix}. \quad (2)$$

$|\mu|$ is the magnitude of the vector μ. The total detected fluorescence can be expressed as (5):

$$F = C \int_{\theta=0}^{\pi} \int_{\phi=0}^{2\pi} S(\theta,\phi)\ N(\theta,\phi) \sin\theta A d\phi\ d\theta. \quad (3)$$

$N(\theta,\phi)$ is the orientation distribution function of the molecules in the adsorption layer. $S(\theta)$ is the collection efficiency of the fluorescence emitted by the molecules of which the transition dipole moment is at an angle θ relative to the z-axis. The collection efficiency does not depend on ϕ, provided that the direction of detection is normal to the interface. C is a constant, which involves the fluorescence quantum yield and the properties of the detection system.

For simplicity, we make the assumption that the orientation of the molecules is isotropic in ϕ and replace $N(\theta,\phi)$ by $N(\theta)/2\pi$. This is true if the correlation length of local ordering along the angle ϕ is much smaller than the dimensions of the illuminated spot. (Correlation in local ordering might rise from lateral interactions between the adsorbed molecules or from anisotropy of the interface in x- and y-directions).

Substitution of Eqs. 1 and 2 in Eq. 3 and integration over ϕ gives for the total detected fluorescence intensity:

$$F = |\mu|^2 C \int_{\theta=0}^{\pi} S(\theta) N(\theta)\ \sin\theta \cdot \left[\frac{1}{2} E_x^2(\Psi) \sin^2\theta + \frac{1}{2} E_y^2(\Psi) \sin^2\theta + E_z^2(\Psi) \cos^2\theta\right] d\theta, \quad (4)$$

in which the dependence of E_x, E_y, and E_z on the polarization angle Ψ of the incident light has been made explicit to emphasize that by measuring F at different polarization angles a transformation of the product $S(\theta)N(\theta)$ to a function of Ψ is obtained. Carrying out the inverse transformation gives the orientation distribution $N(\theta)$, provided that $S(\theta)$ is known. This has been done by Thompson et al. (5) for fluorescent-labeled phospholipids in a supported monolayer by expanding the orientation distribution function in a set of spherical harmonics.

Orientation measurements of adsorbed cytochrome *c*

For TIRF studies of adsorbed cytochrome *c* molecules, the heme group can be used as an intrinsic fluorescent

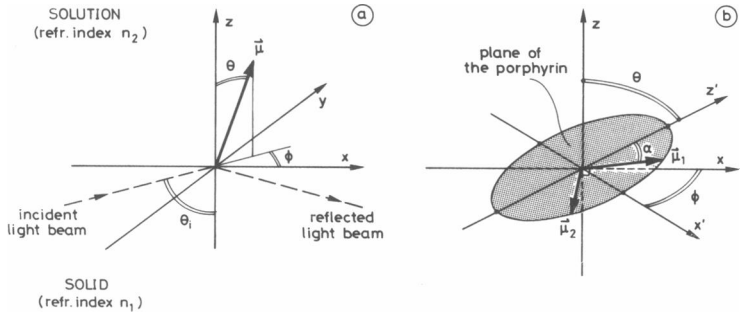

FIGURE 1 Definition of the coordinate system used in the theory. The interface is (parallel to) the xy-plane and the plane of incidence of excitation light is the xz-plane. (*a*) A transition dipole μ with polar and azimuthal angles θ and ϕ interacts with the electric field vector of the evanescent wave; (*b*) the porphyrin ring of cytochrome *c* in which two transition dipole moments lie perpendicular to one another. Their directions are defined by the three orientation angles θ, ϕ, and α (see the text).

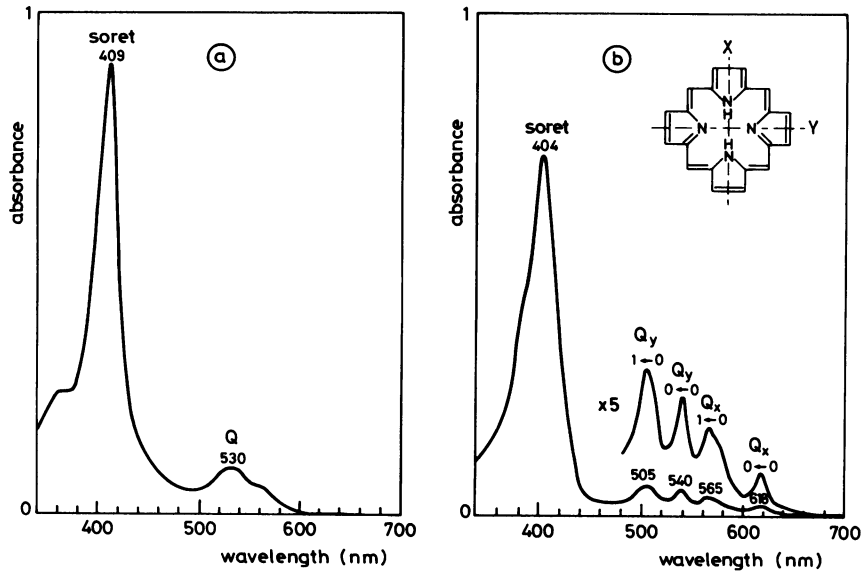

FIGURE 2 The absorption spectra of Fe(III)-cytochrome *c* (*a*) and H_2-cytochrome *c* (*b*) in 10 mM phosphate buffer pH 7 + 0.1 M NaCl. The Q bands represent the two transition dipole moments of the porphyrin ring, which have in the case of H_2-cytochrome *c* a different absorption energy. The 0 ← 0 bands are pure electronic transitions, whereas the 1 ← 0 bands are vibrational transitions.

label, provided that the central Fe atom is removed (or replaced by, e.g., Zn or Sn) (12, 13). Because the three-dimensional structure of cytochrome *c* is known in detail from x-ray diffraction studies (14), the orientation of the heme relative to the rest of the molecule is known.

In Fig. 2 the absorption spectra of the native molecule (Fe-cytochrome *c*) and its free base derivative (H_2-cytochrome *c*) are given. The Q bands represent absorption along two transition dipole moments of the porphyrin ring system, oriented in the plane of the ring and perpendicular to one another (12, 13). In the free base derivative these two transition dipole moments have a different absorption energy due to the presence of two protons on opposite pyrrole rings; this structure has a characteristic four-banded visible spectrum. Unlike Fe-cytochrome *c* in which the emission spectrum of the porphyrin is almost completely quenched by the central metal, the free base derivative is fluorescent. Its emission spectrum is given in Fig. 3.

By exciting the H_2-cytochrome *c* molecule in a Q_X or Q_Y band it is possible to select one of the two transition dipole moments of the porphyrin. (The direction of the transition dipole moment that corresponds to the Q_X band is along the two central protons.) A complication is that the protons in the center of the ring can move over to the other pair of opposite pyrrole rings, resulting in an exchange of the orientations of the two transition dipole moments. Thus, the selected transition dipole current can be in two different orientations, perpendicular to one another: μ_1 and μ_2.

The tautomeric process is much slower than the absorption and emission processes (12, 13). Therefore, the detected fluorescence can be expressed as $S_1\langle(\mu_1 \cdot \mathbf{E})^2\rangle + S_2\langle(\mu_2 \cdot \mathbf{E})^2\rangle$, where S_1 and S_2 are the collection efficiencies of the fluorescence emitted by the two tautomeric forms of the molecule.

Introducing a new orthogonal coordinate system $x'y'z'$ in which the $x'z'$-plane corresponds to the plane of the porphyrin ring system, μ_1 and μ_2 can be described in terms

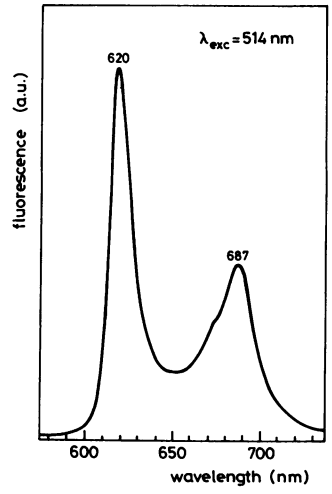

FIGURE 3 Emission spectrum of H_2-cytochrome *c* in 10 mM phosphate buffer pH 7 + 0.1 M NaCl.

of three orientation angles θ, ϕ, and α (see Fig. 1 *b*). The x'-axis is taken in the interface, i.e., in the xy-plane of the previously introduced coordinate system. Now, ϕ is the angle between the x'-axis and the x-axis; θ is the angle between the plane of the porphyrin and the z-axis, i.e., the angle between the z'-axis and the z-axis; α is the angle between μ_1 and the z'-axis. This results in the following expressions for μ_1 and μ_2:

$$\mu_1 = |\mu_1| \begin{pmatrix} \cos\phi \sin\alpha + \sin\theta \sin\phi \cos\alpha \\ -\sin\phi \sin\alpha + \sin\theta \cos\phi \cos\alpha \\ \cos\theta \cos\alpha \end{pmatrix}. \quad (5)$$

$$\mu_2 = |\mu_2| \begin{pmatrix} \cos\phi \cos\alpha - \sin\theta \sin\phi \sin\alpha \\ -\sin\phi \cos\alpha - \sin\theta \cos\phi \sin\alpha \\ -\cos\theta \sin\alpha \end{pmatrix}. \quad (6)$$

As before, it is assumed that the orientation of the adsorbed molecules is isotropic in the x- and y-directions (i.e., in ϕ). The orientation distribution of the molecules is now given by a function $N(\theta,\alpha)$. Now, the following expression for the total fluorescence intensity is obtained:

$$\begin{aligned} F = \frac{1}{2} C \int_{\theta=0}^{\pi} \int_{\alpha=0}^{2\pi} & N(\theta,\alpha) \sin\theta \\ & \cdot \Big[S_1(\theta,\alpha)|\mu_1|^2 \Big\{ \frac{1}{2} E_x^2(\sin^2\alpha + \sin^2\theta \cos^2\alpha) \\ & + \frac{1}{2} E_y^2(\sin^2\alpha + \sin^2\theta \cos^2\alpha) + E_z^2 \cos^2\theta \cos^2\alpha \Big\} \\ & + S_2(\theta,\alpha)|\mu_2|^2 \Big\{ \frac{1}{2} E_x^2(\cos^2\alpha + \sin^2\theta \sin^2\alpha) \\ & + \frac{1}{2} E_y^2(\cos^2\alpha + \sin^2\theta \sin^2\alpha) \\ & + E_z^2 \cos^2\theta \sin^2\alpha \Big\} \Big] d\alpha d\theta. \end{aligned} \quad (7)$$

The factor 1/2 in front of the double integral stems from the reasonable assumption that the absorption transition dipole moment of each molecule is half of the time present as μ_1 and half of the time as μ_2.

Several types of approximations can be made. First, because of the symmetry of the porphyrin ring, it can be assumed that $|\mu_1| \approx |\mu_2| \approx |\mu|$. This implies that the absorption intensity is not sensitive to the angle α, which in fact gives the rotational position of the molecule around the normal of the porphyrin plane. If, furthermore, S_1 and S_2 are taken to be equal and constant, which would be the case if half of the total emitted radiation is collected (5, 15), the detected fluorescence intensity is also not dependent on α. Therefore, in this first approximation it is only possible to retrieve $N(\theta)$, the orientation distribution of the plane of the porphyrin ring. Eq. 7 then simplifies to

$$\begin{aligned} F = \frac{1}{2}|\mu|^2 C \int_{\theta=0}^{\pi} & N(\theta) \sin\theta \\ & \cdot \Big[\frac{1}{2} E_x^2(1 + \sin^2\theta) + \frac{1}{2} E_y^2(1 + \sin^2\theta) + E_z^2 \cos^2\theta \Big] d\theta. \end{aligned} \quad (8)$$

The collection efficiency is now incorporated in the constant C. This expression is similar to Eq. 4. Also in this case, $N(\theta)$ can be obtained by measuring the fluorescence intensity as a function of the polarization angle Ψ.

In the experiments described in this paper, we have only used TE (transverse electric) and TM (transverse magnetic) polarizations of the incident light. For these polarization modes, the evanescent wave has the same polarization direction as the incident light. For the TE polarization mode, **E** is perpendicular to the plane of incidence, which means that $E_x = E_z = 0$. For the TM polarization mode, **E** lies in the plane of incidence and $E_y = 0$. A second type of approximation is to assume that all adsorbed molecules have the same orientation angles θ_0 and α_0. It can be derived from Eq. 7 that the ratio between the fluorescence intensities measured at TM and TE polarization is given by:

$$P \equiv \frac{F_{TM}}{F_{TE}} = \frac{E_x^2}{E_y^2} + \frac{2E_z^2}{E_y^2} \cdot \frac{\cos^2\theta_0}{S + \sin^2\theta_0} \quad (9)$$

with

$$S = \frac{S_1(\theta_o,\alpha_o)\ |\mu_1|^2 \sin^2\alpha_o + S_2(\theta_o,\alpha_o)\,|\mu_2|^2 \cos^2\alpha_o}{S_1(\theta_o,\alpha_o)\ |\mu_1|^2 \cos^2\alpha_o + S_2(\theta_o,\alpha_o)\,|\mu_2|^2 \sin^2\alpha_o}. \quad (10)$$

Assuming that the absorption transition dipole is in the same direction as the emission dipole, which is the case in the orientation measurements described in this paper, the collection efficiencies S_1 and S_2 can be written as

$$S_1(\theta,\alpha) \sim 1 - \gamma \cos^2\theta \cos^2\alpha \quad (11)$$

$$S_2(\theta,\alpha) \sim 1 - \gamma \cos^2\theta \sin^2\alpha. \quad (12)$$

These expressions are comparable to the one used in reference 5. The parameter γ is the so-called dichroic factor and depends on the aperture angle of the detection system. For "ideal" detection the collection efficiencies S_1 and S_2 are equal and constant and $\gamma = 0$. Substitution in Eq. 10 and taking $|\mu_1| = |\mu_2|$ gives for S

$$S = \frac{2 - \gamma \cos^2\theta_o}{1 - \gamma \cos^2\theta_o(\cos^4\alpha_o + \sin^4\alpha_o)} - 1. \quad (13)$$

Note that if the absorption dipole moment is perpendicular to the emission dipole, the term $\cos^2\alpha$ in Eq. 11 should be replaced by $\sin^2\alpha$ and the term $\sin^2\alpha$ in Eq. 12 by $\cos^2\alpha$. This results in a somewhat different expression for S. It

TABLE 1 Comparison of the variation in F_{TM} and F_{TE} with θ_0 for the situation of a constant orientation of the absorption and emission transition dipole moments in a molecule (*a*) and for the case of two possible orientations as in H_2-cytochrome *c* (*b*)

		$\theta_0 = 0°$	$\theta_0 = 90°$
(*a*)	F_{TM}	$E_z^2(1 - \gamma)$	$\frac{1}{2}E_x^2$
	F_{TE}	0	$\frac{1}{2}E_y^2$
(*b*)	F_{TM}	$\frac{1}{4}E_x^2(1 - 2\gamma\cos^2\alpha_0\sin^2\alpha_0) + \frac{1}{2}E_z^2[1 - \gamma(\cos^4\alpha_0 + \sin^4\alpha_0)]$	$\frac{1}{2}E_x^2$
	F_{TE}	$\frac{1}{4}E_y^2(1 - 2\gamma\cos^2\alpha_0\sin^2\alpha_0)$	$\frac{1}{2}E_y^2$

The *F*-values are derived from Eqs. 4, with $S(\theta) = 1 - \gamma\cos^2\theta$ (5), and Eq. 7, with $|\mu_1| = |\mu_2| = |\mu|$, and S_1 and S_2 according to Eqs. 11 and 12, and are divided by $|\mu^2|C$. For "ideal" detection ($\gamma = 0$) the sensitivities of the *F*-values for θ_0 in case *a* and *b* differ a factor 2.

has no further implications for application of this method for orientation measurements.

The fact that H_2-cytochrome *c* has two tautomeric forms resulting in two different orientations for the absorption and emission transition dipoles instead of a constant orientation, reduces the sensitivity of the method by a factor of ~2, somewhat depending on the value of γ. This is illustrated in Table 1, in which the intervals for F_{TM} and F_{TE} are given. For $\theta_0 = 90°$, i.e., the porphyrin parallel to the interface, it makes no difference for the measured fluorescence intensities whether we are dealing with a constant orientation of the transition dipoles or two possible orientations: the dipoles are parallel to the interface anyway. Generally, when a transition dipole moment is normal to the interface, it has no interaction at all with TE polarized radiation ($E_z = 0$). Therefore, for a constant orientation of the absorption transition dipole in the molecules F_{TE} can obtain a zero value. In the case of H_2-cytochrome *c* it is not possible that both μ_1 and μ_2 are normal to the interface, and F_{TE} always has a finite value.

Finally, for a random orientation distribution ($N[\theta,\alpha]$ is constant), again assuming that $|\mu_1| \approx |\mu_2|$, *P* becomes:

$$P_{random} = \frac{E_x^2}{E_y^2} + \frac{2E_z^2}{E_y^2} \cdot \frac{1 - 0.45\gamma}{5 - 1.65\gamma}, \qquad (14)$$

MATERIALS AND METHODS

All chemicals used were analytical reagent grade. Horse-heart cytochrome *c*, Type VI, was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The free base derivative was obtained by modification of Fe(III)cytochrome *c* following the method described by Vanderkooi et al. (12). It was purified by FPLC on a mono S column, gradient 0–1 M NaCl, 20 mM phosphate buffer pH 7. This procedure was repeated until the protein eluted in one sharp peak. The position of this peak was identical with that of Fe-cytochrome *c*, indicating that the structure of the protein was not changed by the modification (see also Discussion). The absorption and emission spectra of the purified derivate are depicted in Figs. 2 and 3 and are identical to those reported in literature (12).

Optical system

In Fig. 4 the optical system is shown. The core of the system is a hemicylindrical quartz prism, of which the center part of the flat backside is in contact with an aqueous solution of cytochrome *c* or pure buffer in a perspex cell. As a light source an Argon laser (Coherent Innova 70, Palo Alto, CA; $\lambda_0 = 514$ nm) was used. For orientation measurements, the Argon laser was used to pump a dye laser (Coherent Radiation Model 590) and the wavelength of excitation was 620 nm.

The laser beam (TE polarized) passes through a number of neutral density filters. To randomize the polarization of the beam, a mistuned $\frac{1}{2}\lambda$ disc was used. After passage through a diaphragm and polarizer, the beam is directed into the hemicylindrical prism. The light beam falls at the interface between prism and solution at an angle of incidence of 75° and is totally reflected. The intensity of the beam was measured with a Coherent optical power meter. For detection of fluorescence a monochromator (82.001; Jarrel-Ash, Waltham, MA) and a photomultiplier (model C 31034A; RCA, Lancaster, PA) were used. The fluorescence was collected through the hemicylindrical prism, perpendicular to the interface and was focused on the monochromator slit using a 50-mm and a 300-mm lens. In front of the monochromator aperture a cut-off filter was placed to block any scattered excitation light. The aperture angle of the detection system was ~30°.

Optically Transparent Electrode (OTE)

The hemicylindrical quartz prism was transformed into an OTE by deposition of a thin, optically transparent film of SnO_2 on its flat backside. The SnO_2 was not doped to minimize the effect of interference of the electromagnetic field with free charge carriers. The thickness of the film was ~200 nm and its resistance amounted to 2.58 kΩ/□. Using conducting glue, a Pt wire was connected to the film and one of the electrode contacts on top of the perspex cell (see Fig. 4). As counter electrode a platinized Pt plate was used. The reference electrode was an

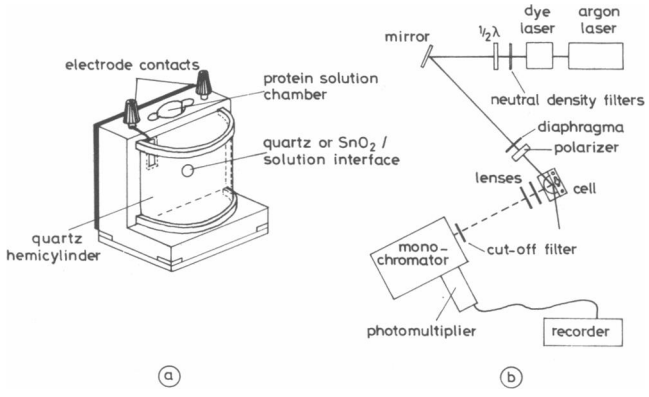

FIGURE 4 Optical system. (*a*) Perspex cell with hemicylindrical quartz prism. (*b*) Configuration of the excitation and detection system relative to the cell with protein solution. For details see the text.

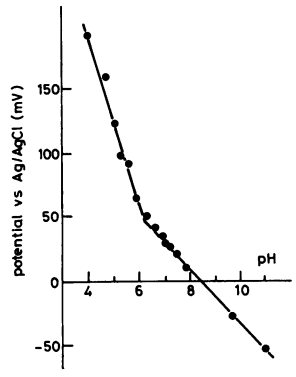

FIGURE 5 Open-circuit potential of the SnO_2-OTE as a function of the pH of the solution.

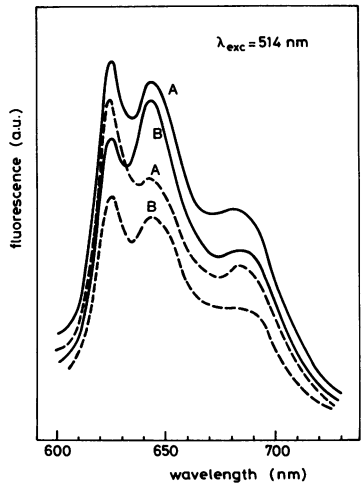

FIGURE 6 Fluorescence spectra of H_2-cytochrome *c* adsorbed at the quartz/solution interface (bulk solution 9 μM protein in 10 mM phosphate buffer pH 7 + 0.1 M NaCl). (—) TM polarized incident light; (- - -) TE polarization. The curves indicated by *A* were recorded 1.5 h after protein injection in the cell, spectra *B* after 2.5 h. (Note: immediately after protein injection only a TM spectrum was recorded, which was fairly the same as the TM spectrum recorded 1.5 h later.) Excitation wavelength: 514 nm; incident light intensity: 0.2 mW for both polarization modes.

Ag/AgCl/3.5 M KCl electrode. Counter and reference electrodes were placed in the solution reservoir of the cell.

If no potential is imposed from outside, the potential of the SnO_2 film is determined by the pH of the solution. In Fig. 5 the open-circuit potentials of the OTE are given as a function of pH. The point of zero charge (pzc) of SnO_2 is pH 4.7 (16). At this pH, the potential difference between the SnO_2 surface and the bulk solution is zero. The pzc corresponds to an open-circuit potential of +143 mV.

The potential of the interface was varied using a simple home-built potentiostat. Because electrochemical reactions at the electrode surface must be avoided, the useful potential range is limited. At a SnO_2 film electrode in 1 N H_2SO_4, below −0.5 V versus Ag/AgCl hydrogen gas evolution takes place and above +2 V oxidation reactions are observed (17). The standard redox potential of Fe-cytochrome *c* is ~0.060 V versus Ag/AgCl (0.262 V/NHE [9]).

RESULTS

Fluorescence of H_2-cytochrome *c* adsorbed at the quartz/solution interface

Before the quartz prism was coated with SnO_2 some experiments were conducted in which cytochrome *c* was adsorbed at the bare quartz surface. A solution of 9 μM H_2-cytochrome *c* in 10 mM phosphate buffer pH7 with 0.1 M NaCl as supporting electrolyte was brought into the cell. Fluorescence spectra at TM and TE polarized incident light (λ_{exc} = 514 nm) were recorded at regular time intervals. (Between the measurements the laser beam was blocked.) In Fig. 6 a number of these spectra are given. Comparison with the emission spectrum of H_2-cytochrome *c* in solution (Fig. 3) shows that for the adsorbed species an additional band is found around 640 nm. The fluorescence intensities at 620 and 687 nm decrease gradually in time, whereas the 640-nm band becomes relatively higher. The latter effect is somewhat stronger for the TM polarization mode than for TE polarization. Similar phenomena have been observed in the emission spectrum of a H_2-cytochrome *c* solution kept in daylight and at room temperature, but the time scale on which the spectrum changed was much larger (weeks).

Replacement of the protein solution in the TIRF cell by pure buffer had hardly any effect on the fluorescence

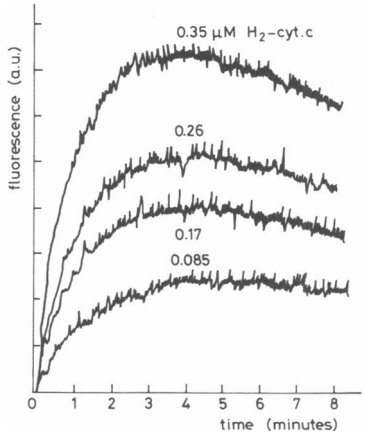

FIGURE 7 Fluorescence intensities of H_2-cytochrome *c* at the quartz/solution interface. At t = 0 the protein was injected into the cell; the bulk concentrations cytochrome *c* (in 10 mM phosphate buffer pH 7 + 0.1 M NaCl) are indicated. Excitation wavelength: 514 nm; detection wavelength: 623 nm; TM polarization; light intensity: 0.4 mW.

intensity, indicating that the recorded fluorescence is almost completely originating from adsorbed molecules and not from molecules in solution. Repeated flushing with buffer or water did not lead to disappearance of the signal; only after overnight contact with a soap solution (Decon 90; Decon Laboratories Ltd., Hove, England) the fluorescence spectrum had completely vanished.

Fig. 7 shows the fluorescence intensity at 623 nm (λ_{exc} = 514 nm) as a function of time at the TM polarization mode. Before the experiments the cell was filled with buffer solution and at t = 0 the protein was added. Because the fluorescence signals did not instantaneously adopt a considerable value upon protein injection, it may again be concluded that only adsorbed molecules contribute significantly to the recorded fluorescence. Furthermore, the initial slopes of the fluorescence-time curves for all cytochrome c concentrations point strongly to a diffusion controlled adsorption process. For the beginning of such an adsorption process, as long as desorption and back-diffusion do not play a role, the equation $\Gamma = 2c_0(Dt/\pi)^{1/2}$ applies (18), with Γ the adsorbed amount per unit area, c_0 the initial concentration, D the diffusion coefficient, and t the adsorption time. The initial slopes of the curves in Fig. 7 are linear with $c_0 t^{1/2}$.

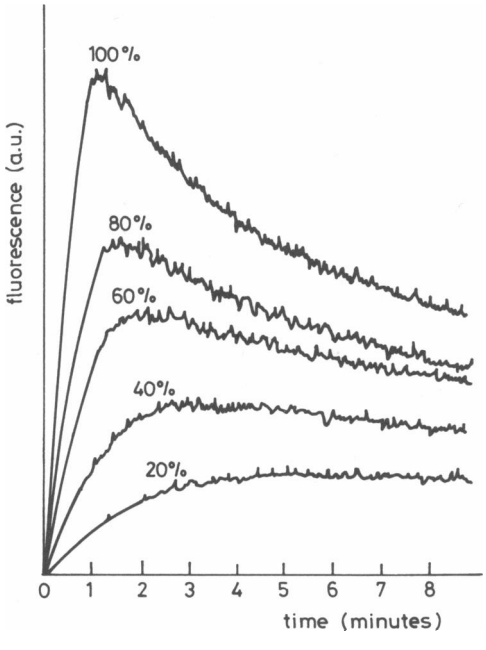

FIGURE 8 Fluorescence intensities of H_2-cytochrome c at the quartz/solution interface. At t = 0 mixed solutions of H_2- and Fe-cytochrome c were injected into the cell; the total protein concentration is 1.5 μM (in 10 mM phosphate buffer pH 7 + 0.1 M NaCl). The percentages of H_2-cytochrome c in the mixtures are indicated. Excitation wavelength: 620 nm; detection wavelength: 680 nm; TM polarization; light intensity: 0.4 mW.

For all protein concentrations the fluorescence decreases after going through a maximum. It was found that by using mixtures of native and free base cytochrome c this phenomenon can be suppressed, which is demonstrated in Fig. 8. The experiments were conducted the same way as before, now using an excitation wavelength of 620 nm, leading to selective excitation of H_2-cytochrome c only (see the absorption spectra of Fig. 2), avoiding interchromophoric interactions between the porphyrin and the heme groups. For the 20% H_2-/80% Fe-cytochrome c mixture no decrease of fluorescence intensity in time was found. The emission spectrum (λ_{exc} = 514 nm) of the adsorbed material still shows an additional band at 640 nm.

Orienting effect of the electric potential of the interface

In the following experiments mixtures of 20% H_2-/80% Fe-cytochrome c were used. The proteins were dissolved in 25 mM phosphate buffers (pH 4 or 10) with 0.1 M KCl as the supporting electrolyte. The total cytochrome c concentration was 2.7 μM. Excitation took place at 620 nm and the fluorescence was detected at 687 nm; in this way the absorption dipole is in the same direction as the emission dipole (12).

Typical results on the adsorption of cytochrome c at the SnO_2-OTE at pH 10 are displayed in Fig. 9. This pH is the isoionic point of the protein (19), so the molecules bear no net charge. At t = 0 protein was added to the buffer solution in the cell. At this stage the potentiostat was not yet switched on, so the potential of the SnO_2/solution interface is determined by the pH (see Fig. 5).

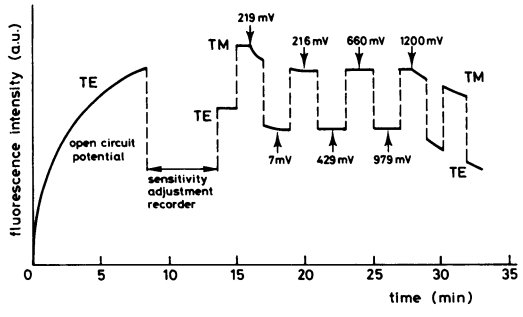

FIGURE 9 Fluorescence intensities of H_2-cytochrome c at the surface of the SnO_2-OTE for TM and TE polarized incident light. At t = 0 a mixed solution of 20% H_2-/80% Fe-cytochrome c was injected into the cell; the total protein concentration is 2.7 μM (in 25 mM phosphate buffer pH 10 + 0.1 M KCl). At t = 16 min the potentiostat was switched on and the potential of the SnO_2-OTE was stepwise varied with intervals of several minutes. The applied potentials versus Ag/AgCl are indicated. Excitation wavelength: 620 nm; detection wavelength: 687 nm; ratio of light intensities: TM/TE = 0.84.

The surface charge density is negative and in the order of tens of microcoulombs per square centimeter (20). After 8 min the sensitivity of the recorder had to be adjusted. In the mean time the fluorescence intensity reached a plateau value and the signal was recorded for TM and TE polarization. The intensity ratio P was found to be 1.7 (corrected for the difference between the light intensities at the TM and TE polarization modes).

At t = 16 min the potentiostat was switched on at a potential of +219 mV versus Ag/AgCl, resulting in a slight decrease of the fluorescence intensities, but P did not change significantly. Subsequently, the potential was stepwise varied from +7 to +979 mV. Up to this potential no changes in the fluorescence intensities for both polarization modes are observed. Therefore, it is concluded that in this potential range no changes take place in adsorbed amount nor in the orientation of the adsorbed cytochrome c molecules. At a potential of +1.2 V the fluorescence intensities decreased and an electrolysis current was observed. As for the quartz surface, it was not possible to remove the protein from the SnO_2 surface by flushing with buffer solution or water. After overnight contact with a solution of 0.5% sodium dodecyl sulphate in 0.5 M NaOH the fluorescence signal had disappeared.

Similar experiments were performed at pH 4. At this pH the SnO_2 surface has a low positive surface charge of $\sim 1\ \mu C/cm^2$ (20) and the protein molecule has a net proton charge of +14 (19). Also in this case the plateau in fluorescence intensity was reached in ~10 min. P was found to be to ~1.3. After switching on the potentiostat at a potential of ~−200 mV, the fluorescence signal dropped quickly and a small electrolysis current was observed, of which the latter phenomenon might be due to reduction of Fe(III)cytochrome c to Fe(II)cytochrome c. Between 0 and +1 V the fluorescence intensities for both polarization modes were constant and P was ~1.4.

When keeping the potential of the interface at +800 mV versus Ag/AgCl during protein injection into the cell the plateau in fluorescence intensity at pH 10 was reached within 5 min and P amounted to 1.4. Again, both the level of the fluorescence intensities and the intensity ratio P stayed the same upon variation of the potential from +800 to −80 mV. At pH 4, the fluorescence intensity reached its plateau in ~8 min and for P a value of 1.3 was found. Subsequent variation of the potential from +800 to 0 mV did not influence this value.

An experiment was conducted in which the protein solution 25 s after injection was replaced by pure buffer; adsorption took place at pH 10 and at the open-circuit potential of the OTE. This results in a low surface coverage: see Fig. 9 and compare the fluorescence intensity after 25 s with its plateau value. In this case P was found to be 2.0. The potentiostat was switched on and the interfacial potential was varied from 0 to +1 V. This resulted in a slight decrease of the fluorescence intensities, but P remained the same.

TABLE 2 Fluorescence intensity ratios P and calculated orientation angles θ_0 for cytochrome c adsorbed at the SnO_2/solution interface under various adsorption conditions

Adsorption conditions				
pH	surface charge or applied potential	$\lvert E\rvert \times 10^{-6}$*	P	$\theta_0(S = 1)$
		(V/m)		
10	$< -10\ \mu C/cm^2$	>12	1.7	38°
			2.0‡	34°
10	+800 mV	>30	1.4	44°
4	$+1\ \mu C/cm^2$	1	1.3–1.4	44–45°
4	+800 mV	>30	1.3	45°

(Ionic strength ≈ 0.1 M). For explanation see the text.
*Estimated electric field strength at 3 nm from the bare interface.
‡<50% surface coverage.

In Table 2 the results of the orientation measurements are summarized. The tabulated values of the electric field strength $/E/$ (not to be confused with the electric field vector of the excitation light) at a distance of 3 nm from the interface have been estimated from the surface charge densities or applied potentials using the diffuse double layer theory and are for the bare interface. The components E_x, E_y, and E_z of the evanescent field have been calculated according to the matrix method of Abeles (11, 21), using the following parameters: n(quartz) = 1.46191, $n(SnO_2)$ = 1.997 (22), n(water) = 1.33317, λ_{exc} = 620 nm, angle of incidence = 75°, thickness of the SnO_2-layer = 200 nm. This gives a penetration depth (defined as the distance required for the electric field amplitude to fall off to e^{-1} of its value at the interface) of 212 nm. Because the cytochrome c molecules have a diameter of only ~3 nm, the amplitude of the evanescent wave is approximately constant over the adsorption layer. At the interface E_x = 0.3622, E_y = 0.8413, and E_z = 1.0989 per unit incoming light at the TM or TE polarization mode. The tabulated orientation angles θ_0 have been calculated from P using Eq. 9 and S = 1. Note that it is not possible to distinguish between θ_0 and $180° - \theta_0$.

From the data in reference 15 and our own calculations it was found that for a detection aperture angle of 30° the dichroic factor γ is ~0.86. Combinations of θ_0 and α_0 that suit various values of P according to Eqs. 9 and 13, are given in Fig. 10.

DISCUSSION

The structure of the native cytochrome c molecule in solution is very stable; between pH 3 and pH 12 no

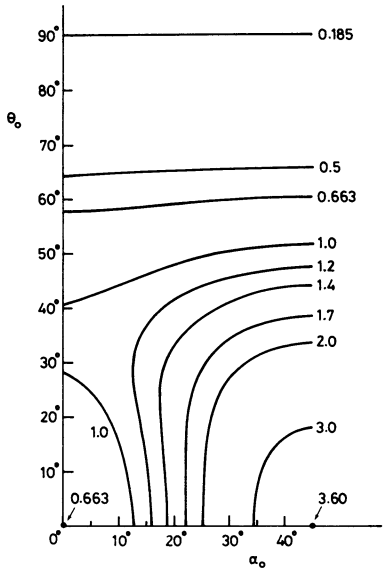

FIGURE 10 Combinations of the orientation angles α_0 and θ_0 of the porphyrin of cytochrome *c* that suit particular values of *P* according to Eqs. 9 and 13 with $E_x = 0.3622$, $E_y = 0.8413$, $E_z = 1.0989$, and $\gamma = 0.86$. The values for *P* are indicated. The curves are symmetrical around the axes $\theta_0 = 90°$ and $\alpha_0 = n.45°$ ($n = 0,1...7$). For a flat orientation of the porphyrin ($\theta_0 = 90°$), *P* is the same for all values of α_0 ($P = E_x^2/E_y^2 = 0.185$). The maximum value for $P = E_x^2/E_y^2 + 2E_z^2/E_y^2 = 3.60$, which is found for $\theta_0 = 0°$ and $\alpha_0 = 45°$.

structural changes of importance takes place (23, 24). The heme group is covalently attached to the peptide chain and has a constant orientation in the molecule. It has been shown from tryptophan fluorescence and CD measurements (12, 25, 26) that there are no significant structural differences between the native and the free base cytochrome *c* molecules. This is affirmed by our finding that in the FPLC chromatograms the peaks of Fe- and H_2-cytochrome *c* are at exactly the same position. However, H_2-cytochrome *c* has a somewhat lower structure stability toward guadinin-HCl and heat denaturation treatments and it has been observed that it undergoes deterioration in the presence of light (12, 25).

Protein molecules may undergo structural rearrangements upon adsorption, depending on the contribution from intramolecular hydrophobic bonding relative to those from other interactions, to the overall stabilization of the structure in solution. In reference 27 an analysis has been made of the structure determining factors for the proteins HPA (human plasma albumin) and RNase (ribonuclease). It leads to the conclusion that RNase is much less able than HPA to adapt its structure at sorbent surfaces. Cytochrome *c* resembles RNase with respect to a number of structure determining properties, e.g., molecular weight, size and shape, hydrophobicity, and Gibb's energy change for transition to the unfolded state (24, 27–29). Furthermore, we found that the adsorption behavior of cytochrome *c* on hydrophilic and hydrophobic surfaces is very much like of that of RNase (unpublished results); e.g., contrary to HPA both of these proteins do not adsorb at hydrophilic surfaces if the charge signs of protein and surface are the same. These facts strongly indicate that the native cytochrome *c* molecule is likely to retain its structure upon adsorption, which is corroborated by the finding that cytochrome *c* in the adsorbed state has, within experimental error, still its crystallographically determined dimensions (30).

However, we are not quite sure that the free base derivate does not change its conformation during the adsorption process. On the one hand, the observed spectral changes in the fluorescence of H_2-cytochrome *c* after adsorption at the quartz surface could point to a change in structure, resulting in an exposure of the porphyrin to the solvent and a Stokes shift of the 620-nm emission band. Short range interactions between exposed porphyrin rings (e.g., excimer formation [31]) are less probable as a cause for the spectral changes because also for low ratio H_2-Fe-cytochrome *c* mixtures a clear 640-nm emission band was found. The fact that for free base cytochrome *c* solutions kept at room temperature and in daylight the same spectral changes are found, could imply that they are due to denaturation. On the other hand, neither heat and guadinin-HCl denaturation treatments nor exposure to extreme light intensities lead to significant changes in the shape of the emission spectrum of the free base derivative in the 600–700 nm wavelength range, and certainly not to the development of an emission band at 640 nm. Therefore, we still do not have an adequate explanation for this phenomenon.

The decrease in total fluorescence intensity at relatively short times (see Fig. 7) is probably due to interchromophoric interactions because it can be suppressed by using mixtures of Fe- and H_2-cytochrome *c*. Coulombic resonance interactions according to the Förster mechanism leading to lower fluorescence intensities can take place if the distance between chromophores is <6 nm and if there is an overlap in the absorption and emission spectra (31). These conditions are fulfilled for a fully-packed monolayer of free base cytochrome *c* or for a patch-wise adsorption of the protein. The latter is probably the case because from adsorption isotherms of cytochrome *c* on glass powder at pH 7 and 0.1 M ionic strength (unpublished results obtained in our laboratory) it was found that at the here applied H_2-cytochrome *c* concentrations the surface is not yet fully covered with the protein: at 0.085 μM the surface coverage is ~10%, at 0.35 μM ~25%.

Due to an inhomogeneous distribution of charged groups, horse heart cytochrome *c* has a relatively strong electric dipole moment, which amounts to 325 Debye

(1.08×10^{-27} Cm) at pH 7 (32). It has been suggested that the direction of this dipole is of physiological importance: it would cause the molecule to orient itself in the electric fields of its redox partners. The electrical field strengths close to the surfaces of these redox partners (cytochrome *c* oxidase and cytochrome *c* reductase) are in the order of 20×10^6 V/m (32). A rotation of 180° from the physiological "active" orientation to the antiparallel orientation would require an energy expenditure of ~10 kT. Because in most of our experiments the electric field strength of the SnO_2 surface is in the same order (Table 2), we expect an electrically induced orientation in the adsorption layer.

The measured P values indeed point to a certain orientation: if the molecules were randomly packed P would be ~0.77, calculated from Eq. 14. Furthermore, it is possible to influence the orientation of the porphyrin ring by variation of the charge of the interface because P is different for different adsorption conditions (pH, applied potential).

In all experiments with the SnO_2-OTE it was found that variation of the potential after adsorption had taken place could not induce a change in the value of P. It seems that the interfacial conditions at the moment of adsorption of a cytochrome *c* molecule determine its orientation at the interface and that the molecule after it has been adsorbed does not change its orientation anymore, because of its strong binding to the interface (also reflected in the difficulties encountered to clean the OTE again). This would imply that a molecule approaching the interface can orient itself only before actual attachment. For the ionic strength of the solutions applied here (~0.1 M), the Debye length of the electrical double layer is ~1 nm. Therefore, the electric field of the interface can be sensed by the molecules only at a distance of a few nanometers. However, from the diffusion constant of cytochrome *c* in water (1.14×10^{-10} m^2/s [33]) and the rotation correlation time of the molecules (5 ns [26]), it is found that the time of diffusion through the double layer is long enough to rotate to the most favorable orientation.

The circumstances at the interface for the first adsorbing molecules differ from those for molecules that arrive at the surface in a later stage. For example, as the surface becomes covered with protein molecules, the electric field strength at the solution side of the adsorption layer decreases (27) and the size of the empty spaces in the adsorption layer become limiting for orientation. Because a significant difference between the P values for a partly and a fully covered SnO_2 surface at pH 10 was observed, it is concluded that indeed the average orientation of the first adsorbing molecules is different from that of the complete adsorption layer. At pH 10 the SnO_2 surface is negatively charged and the electric field vector **E** points toward the interface. When a positive potential of 800 mV is applied, the direction of the electric field is just the other way round. If in both cases the alignment of the dipole moments along the electric field would be complete, one would expect to measure the same P value, because it is not possible to discriminate between θ_0 and $180° - \theta_0$. Nevertheless, this was not found, which can have several causes: (*a*) the electric field strength of the surface at the open-circuit potential is lower than for an imposed potential of 800 mV and therefore orienting effects other than dipole/electric field interactions are relatively more important; (*b*) the charge distribution of the cytochrome *c* molecule and therefore the direction of its dipole moment is affected by the electric field of the surface; and (*c*) structural changes of the free base cytochrome *c* molecule could play a role.

At pH 4 the SnO_2 surface is almost in its pzc and the electric field strength is relatively low. It is therefore remarkable that P has approximately the same value as when a potential of 800 mV is applied.

The measured values of P give only possible combinations of the (mean) orientation angles θ_0 and α_0 of the absorbed molecules, represented by the curves in Fig. 10. The accuracy of these curves depends on the correctness of the assumptions made and the degree of uncertainty in the amplitude of the evanescent wave. In particular, the assumption that S_1 and S_2 do not depend on ϕ is in this case not entirely correct, because of the anisotropic focusing effect of the hemicylindrical prism in the detection path of the experimental set-up. The uncertainties in E_x, E_y, and E_z are mainly due to the fact that the refractive index and the thickness of the semi-conducting SnO_2 layer are not exactly known. For the moment, we suffice to review the experimental P values on the basis of the known direction of the dipole moment of horse heart cytochrome *c* at pH 7 (32). Unfortunately, data at other pH values are not (yet) available. At pH 7 the angle between the dipole moment and the plane of the heme is 33°; the angle between the projection of the dipole moment in the heme plane and one of the transition dipole moments of the heme is ~14°. If these data also apply to the adsorbing free base cytochrome *c* molecule, a perfect alignment of the electric dipole along the electric field of the surface would yield under our experimental conditions a value of $P = 1.2$. This result is quite satisfactory.

In conclusion, we have demonstrated that TIRF can be a useful tool in examining the orientation of adsorbed proteins with structure-stable conformations. Studying the orientation as a function of the potential of the interface can give insight into the role of electrostatic interactions in the adsorption process and may prove to be of practical use in cases where a specific orientation of certain proteins is wanted, e.g., in the deposition of immunoglobulins at the surface of biosensor devices.

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