Determination of the Orientation Distribution of Adsorbed Fluorophores Using TIRF. II. Measurements on Porphyrin and Cytochrome *c*

Martin A. Bos and J. Mieke Kleijn
Department of Physical and Colloid Chemistry, Agricultural University, 6700 EK Wageningen, The Netherlands

ABSTRACT The theory for determination of the orientation of adsorbed fluorescent molecules using total internal reflection fluorescence, as explained in part I of this series, is illustrated by measurements on adsorbed tetramethylpyridinium porphyrin (H_2TMPyP) and porphyrin cytochrome *c* molecules. The results are encouraging, although for porphyrin cytochrome *c* the scatter in the obtained order parameters is substantial. For H_2TMPyP molecules adsorbed on glass the orientation distribution depends on the solution concentration. At low concentration, the H_2TMPyP molecules are more or less randomly oriented, whereas at high concentrations a broad distribution around an angle of 46° between the porphyrin plane and surface was found. For cytochrome *c* adsorbed on glass and indium tin oxide it was impossible to interpret the data in terms of orientation distributions because of the scatter in the results. The total fluorescence as a function of the polarization angle Ψ of the incident light beam corresponds to an average angle between the porphyrin group and the surface of 30°-40°. Despite the strong electric dipole moment of the protein, the orientation distribution seems to be independent on the (imposed) electrical potential of the interface.

INTRODUCTION

In the first paper of this series (Bos and Kleijn, 1994) the theory underlying orientation measurements on fluorophores at solid surfaces using total internal reflection fluorescence (TIRF) was explained. The aim of the present paper is to demonstrate the applicability of the theory by measurements on adsorbed tetramethylpyridinium porphyrin (H_2TMPyP) and porphyrin cytochrome *c* molecules.

For easy reference we repeat here very briefly the principle of the method. Fluorescent molecules near an optically transparent solid surface are selectively excited by an evanescent field. Analysis of the resulting fluorescence intensity and polarization as a function of the polarization angle Ψ of the incident light beam provides a number of order parameters of the orientation distribution of the fluorophores. In the case of molecules with a porphyrin ring as the fluorescent group, the orientation can be described in terms of three angles, θ, ϕ, and α. The angles θ and ϕ define the orientation of the porphyrin ring with respect to the interface; θ is the tilt angle between the porphyrin ring and the surface. The angle α gives the rotational position of the molecule around the normal of the porphyrin ring. By assuming isotropy in the *xy* plane (the interface) the angle ϕ can be eliminated from the equations describing the fluorescence signal as a function of Ψ. For excitation of the porphyrin group in the $Q_y(1, 0)$ absorption band, the fluorescence signal is not sensitive to the angle α, so that only the orientation in the angle θ is determined. The obtained order parameters are

$$\langle P_2 \rangle = \frac{1}{2}(3\langle \cos^2\theta \rangle - 1),$$

$$\langle P_4 \rangle = \frac{1}{8}(35\langle \cos^4\theta \rangle - 30\langle \cos^2\theta \rangle + 3),$$

where the angle brackets $\langle\ \rangle$ denote an average over all abundant orientations.

Unlike native cytochrome *c* in which the emission of the porphyrin is almost completely quenched by the central Fe atom, porphyrin cytochrome *c* is fluorescent (Vanderkooi and Erecinska, 1975). It has been shown from tryptophan fluorescence and CD measurements that there are no significant structural differences between the native and the porphyrin forms of cytochrome *c* molecules (Fisher et al., 1973; Vanderkooi and Erecinska, 1975; Vos et al., 1987). This is confirmed by the observation that in FPLC chromatograms the peaks of native and porphyrin cytochrome *c* are at exactly the same position (Fraaije et al., 1990).

Because of an inhomogeneous distribution of charged groups, cytochrome *c* has a relatively strong electric dipole moment, which amounts to 1.08×10^{-27} Cm (325 D) at pH 7 (Koppenol and Margoliash, 1982). 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 field of its redox partners, cytochrome *c* oxidase and cytochrome *c* reductase. In the present study we have tried to affect the orientation of adsorbed cytochrome *c* molecules by varying the electric potential of the sorbent surface.

MATERIALS AND METHODS

All chemicals used were analytical reagent grade. Water was purified by a millipore purification system, involving reversed osmosis and subsequent percolation through charcoal and a mixed-bed ion exchange resin. Tetra-

Received for publication 3 October 1994 and in final form 11 January 1995.
Address reprint requests to Dr. J. Mieke Kleijn, Department of Physics and Colloid Chemistry, Agricultural University, P.O. Box 8038, 6700 EK Wagenigen, The Netherlands. Tel.: 31-8370-82279; FAX: 31-8370-83777; E-mail: fysko@fenk.wau.nl.

0006-3495/95/06/2573/07 $2.00

methylpyridinium porphyrin ($H_2TMPyPI_4$) was obtained from Strem Chemicals GmbH (Kehl, Germany) and horse heart cytochrome *c* from Sigma (St. Louis, MO) (type III).

Porphyrin cytochrome *c* was prepared from the native protein following a method described by Flatmark and Robinson (1968). The derived protein was dissolved in 0.1-M ammonium acetate buffer pH 5 and dialyzed against the same buffer for 24 h. Subsequently, the protein was lyophilized and stored at −18°C.

H_2TMPyP and cytochrome *c* concentrations in solution were determined spectrophotometrically (H_2TMPyP at 424 nm, molar extinction coefficient 220×10^3 M^{-1} cm^{-1} (Kalyanasundaram and Neumann-Spallart, 1982); cytochrome *c* at 404 nm, molar extinction coefficient 81×10^3 M^{-1} cm^{-1} (Vanderkooi et al., 1976). The absorption and emission spectra of H_2TMPyP and porphyrin cytochrome *c* are shown in Figs. 1 and 2.

For TIRF measurements H_2TMPyP was adsorbed on a glass plate. Porphyrin cytochrome *c* was adsorbed on either a glass plate or an indium tin oxide (ITO) film (thickness 120 nm) deposited on glass. Adsorption took place in a laminar flow cell in the TIRF setup, at a flow rate of 2.0×10^{-8} m^3 s^{-1}. In the flow cell a connection was made to impose an electrical potential to the semiconducting ITO film, and a counterelectrode was mounted. A reference electrode (Ag/AgCl/saturated KCl) was kept outside the flow cell and placed into a small beaker in contact with the solution inside the flow cell.

Optical system

In Fig. 3 a schematic diagram of the TIRF apparatus is shown. A sealed head pulsed nitrogen laser (model VSL-337ND, Laser Science, Inc., Cambridge, MA) with an emission wavelength of 337 nm was used to pump a dye laser module (model DLM-220, Laser Science, Inc.). The dye laser produces pulses of 3 ns with an energy of 50 μJ per pulse; the average power is 1 mW. The beam from the dye laser (wavelength 514 nm, polarized horizontally) passes through a broadband polarization rotator (model PR-550, Newport Corp., Irvine, CA), and a focusing plano–convex lens and enters the 75° Dove glass prism of the TIRF cell normal to one of the small beveled faces. A glass plate is optically coupled to the large face of the prism, using immersion oil (Brunschwig Chemie, Amsterdam, $n = 1.515$ at 20°C) as the refractive matching fluid. The glass surface (or the ITO film on one side of the glass plate) serves as one face of the flow chamber for adsorption. The light beam is totally reflected at the glass (or ITO)/solution interface. The area of the reflection spot is approximately 1 mm^2. The reflected laser beam is absorbed by a light trap. Part of the fluorescence light emitted by fluorophores at the solid/liquid interface is transmitted through the prism and is collimated and condensed by two plano–convex lenses into a spectrograph (model 1233, EG&G Princeton Applied Research, Princeton, NJ) with a vertical grating. As a consequence light polarized perpendicularly with respect to the plane of incidence with a wavelength between 600 and 800 nm is detected a factor of 1.25 better than by parallel polarized light. In comparing experimental fluorescence data with theoretical curves this has been taken into account. The overall aperture angle of the detection system is approximately 10°. The detection is gated. A polarization filter is used for separate detection of the components of the fluorescence polarized perpendicular and parallel to the plane of incidence. A cutoff filter is placed in front of the spectrograph to block scattered excitation light. An optical multichannel analyzer (model 1463, EG&G Princeton Applied Research) is used for data acquisition and analysis.

For the determination of the orientation of the plane of the porphyrin ring, excitation of the adsorbed molecules took place at a wavelength of 514 nm, i.e., in the Q_y (1, 0) absorption band (see Figs. 1 and 2). At various polarization angles Ψ of the incident light beam, the fluorescence spectrum was recorded 100 times, added up, and integrated between 600 and 800 nm, giving the fluorescence signal $F(\Psi)$.

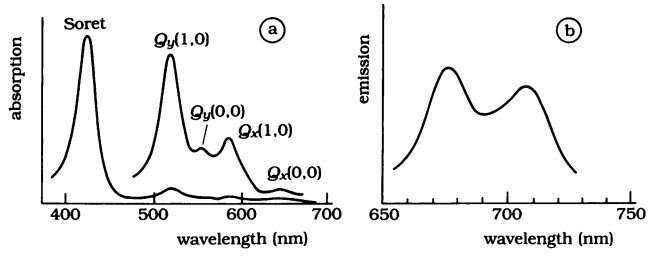

FIGURE 1 (*a*) Absorption spectrum of H_2TMPyP in 10-mM phosphate buffer pH 7. (*b*) Emission spectrum of H_2TMPyP adsorbed on glass as determined by TIRF. Adsorption took place from 10-mM phosphate buffer pH 7. Excitation wavelength 514 nm.

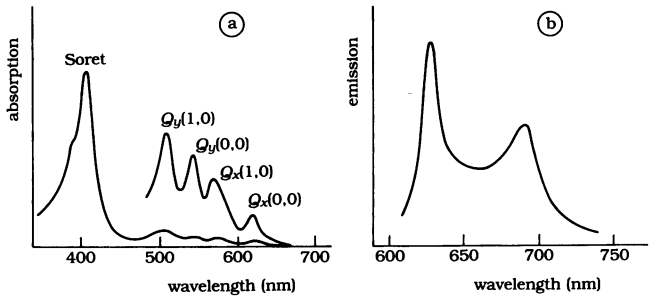

FIGURE 2 (*a*) Absorption spectrum of porphyrin cytochrome *c* in 10-mM phosphate buffer pH 7. (*b*) Emission spectrum of porphyrin cytochrome *c* adsorbed on glass as determined by TIRF. Adsorption took place from 10-mM phosphate buffer pH 7. Excitation wavelength 514 nm.

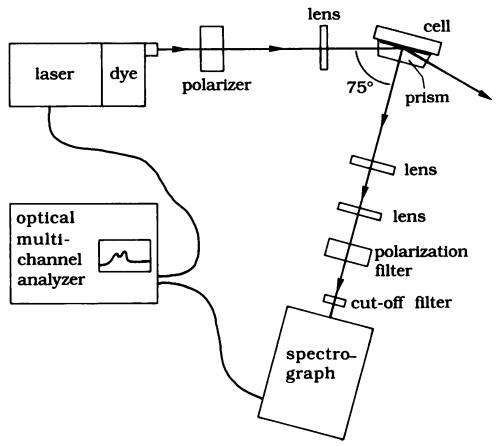

FIGURE 3 Schematic diagram of the TIRF setup.

RESULTS

Orientation measurements on adsorbed tetramethylpyridinium porphyrin molecules

In Fig. 4 (*a*) $F(\Psi)$ is shown as obtained for H_2TMPyP molecules adsorbed on glass from solutions of 1- and 10-mg/l H_2TMPyP in water. The experimental points are fitted (least squares) according to $F(\Psi) = A + B \cos^2 \Psi$. The good fit proves that the fluorescence stems from molecules that are excited by the evanescent field and not by stray light of the incident laser beam (Bos and Kleijn, 1994).

In Fig. 4 *b* theoretical curves for $F(\Psi)$ are given for sharp distributions with $\theta = 0°$, 45°, and 90° and for a random orientation. These curves have been calculated according to

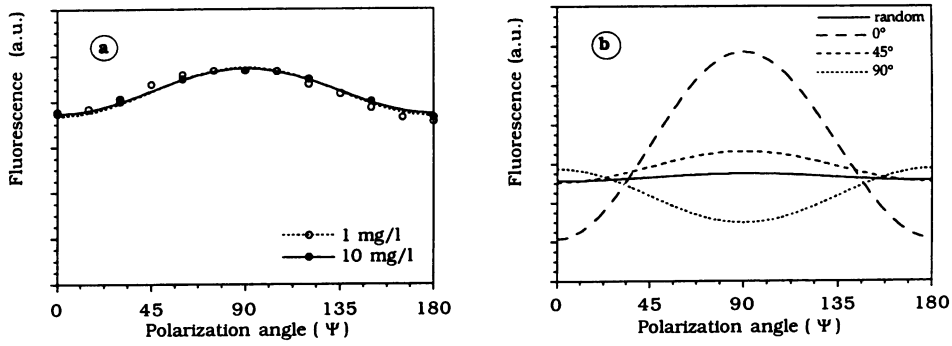

FIGURE 4 Fluorescence signal of H_2TMPyP adsorbed on glass as a function of the polarization angle Ψ of the incident light beam. (*a*) Experimental points for adsorption from solutions of 1- and 10-mg/l H_2TMPyP in 10-mM phosphate buffer pH 7. The curves through the points are obtained by fitting with $F(\Psi) = A + B \cos^2(\Psi)$. (*b*) Calculated curves for a random distribution and sharp distributions with orientation angle $\theta = 0°$, 45°, and 90°.

Eq. 11 of Bos and Kleijn (1994), using a value of 0.98 for the dichroic factor γ, corresponding to the aperture angle of the detection system in the experimental setup (Burghardt and Thompson, 1984). The components of the electric field vector of the evanescent field, ϵ_x, ϵ_y, and ϵ_z, have been calculated using the equations given by Harrick (1967), with refractive indices of 1.518 for glass and 1.333 for the solution phase and an angle of incidence of 75°. In the adsorption layer (refractive index n_p) the normal component (ϵ_z) differs from that in the solution by a factor $(1.333/n_p)^2$; the tangential components (ϵ_x and ϵ_y) are continuous across the interface (Harrick, 1967). Taking for n_p a value of 1.4, a common value for organic compounds, the values of the electric field components in the adsorption layer become $\epsilon_x = 0.464$, $\epsilon_y = 1.082$, and $\epsilon_z = 1.009$. The experimental results depicted in Fig. 4 (*a*) correspond to $F(\Psi)$ curves for sharp distributions with $\theta \approx 47°$. More information on the orientation distribution of the porphyrin molecules cannot be obtained from $F(\Psi)$ alone (Bos and Kleijn, 1994).

To determine the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ of the orientation distribution, the parallel and perpendicular polarized components of the fluorescence at $\Psi = 0°$ and $\Psi = 90°$ have been measured, i.e., $F_\parallel(0°)$, $F_\perp(0°)$, $F_\parallel(90°)$, and $F_\perp(90°)$. Results are shown in Fig. 5. All ($\langle P_2 \rangle$;$\langle P_4 \rangle$) combinations derived were found to be within their physical boundaries. Although there is some scatter in the obtained order parameters, especially in $\langle P_4 \rangle$, a trend in the orientation distribution with porphyrin concentration in solution can be clearly distinguished. The values for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for adsorption from a solution with a porphyrin concentration of 1 mg/l are approximately 0.2 and 0.0, respectively. In the case of concentrations of 10 and 100 mg/l, $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are approximately 0.2 and −0.2, respectively. For adsorption from a porphyrin solution of 0.1 mg/l reliable order parameters could not be derived, because the fluorescence signals were too weak.

Orientation distribution functions corresponding with the average values for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for 1, 10, and 100 mg/l are shown in Fig. 6. These distributions are calculated by the maximum-entropy method (see Bos and Kleijn, 1994). At 1 mg/l the orientation distribution is almost random. At concentrations of 10 and 100 mg/l the adsorbed molecules show clearly a preferential orientation angle θ of 45°–46°. The value of $\langle P_4 \rangle$ (−0.2) indicates a sharper distribution than at 1 mg/l. For a really sharp distribution with $\theta = 45°$, $\langle P_4 \rangle$ would amount to −0.41.

Orientation measurements on adsorbed porphyrin cytochrome *c* molecules

Experiments similar to those for H_2TMPyP have been performed for adsorbed porphyrin cytochrome *c* molecules. As

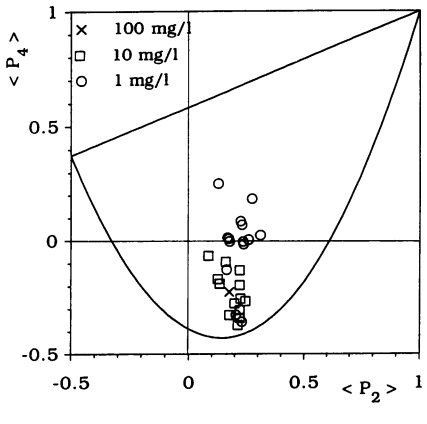

FIGURE 5 Order parameters for H_2TMPyP adsorbed on glass from solutions of various porphyrin concentrations. The physical boundaries of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are indicated by solid lines.

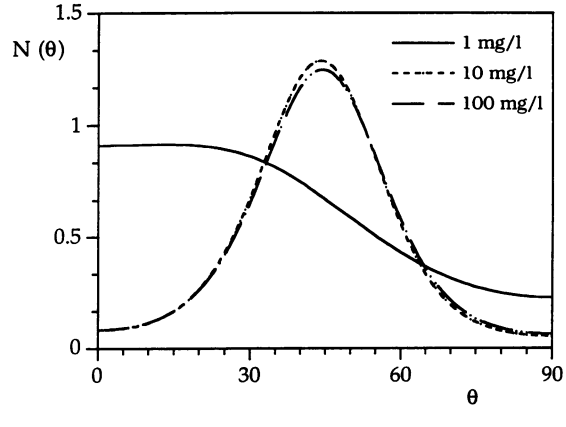

FIGURE 6 Orientation distribution functions of H_2TMPyP adsorbed on glass, corresponding to the average values for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for adsorption from solutions with porphyrin concentrations of 1, 10, and 100 mg/l and calculated following the maximum-entropy method.

sorbent surfaces glass and ITO were used; in some cases an electrical potential was externally imposed on the ITO/solution interface.

Fig. 7 shows the measured and theoretical fluorescence signals as a function of the polarization angle Ψ of the incident light beam. The solid curves through the experimental points in Fig. 7 (*a*) and 7(*b*) represent the best fit of $A + B \cos^2 \Psi$. The theoretical curves for glass as the sorbent surface (Fig. 7 (*c*)) are calculated using the same parameters as before. (Thus, for the protein adsorption layer also, a refractive index of 1.4 has been used to determine ϵ_z in this layer (Ivarsson et al., 1985). In the case of ITO on glass (Fig. 7 (*d*)), the components of the electric field vector of the evanescent field were obtained by Hansen's method based on the matrix formalism of Abeles (Hansen, 1968), with a thickness of 120 nm and a refractive index of 1.90 for the ITO film; the other parameters used are the same as before. This results in $\epsilon_x = 0.426$, $\epsilon_y = 0.600$, and $\epsilon_z = 0.927$ in the protein adsorption layer. The measured $F(\Psi)$ curves given in Fig. 7 (*a*) and 7 (*b*) correspond to $F(\Psi)$ curves for sharp distributions with $\theta \approx 38°$ and $\theta \approx 31°$, respectively.

The experimental results in Fig. 7 are for adsorption from a solution of 10-mg/l porphyrin cytochrome *c* in 10-mM phosphate buffer pH 7. For adsorption from solutions with other concentrations protein (varying from 1 to 100 mg/l) approximately the same $F(\Psi)$ curves were found. Imposing an external potential at the ITO/solution interface (in the range of −400 to +800 mV versus an Ag/AgCl reference electrode) during or after adsorption of the protein did not significantly affect the shape of $F(\Psi)$.

To obtain more specific information on the orientation distribution, the fluorescence signals $F_{\parallel}(0°)$, $F_{\parallel}(90°)$, $F_{\perp}(0°)$, and $F_{\perp}(90°)$ have been measured under various experimental conditions. Most of the values derived for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are shown in Fig. 8; some values were found to be outside the axes of this plot. There is no clear correlation with the protein concentration of the solution from which adsorption took place or with the interfacial potential imposed during or after adsorption. The $\langle P_2 \rangle$ values found within the physical boundaries of the order parameters are all between −0.05 and +0.5 (corresponding to orientation angles between 35 and 55°), whereas $\langle P_4 \rangle$ is found to be rather undefined.

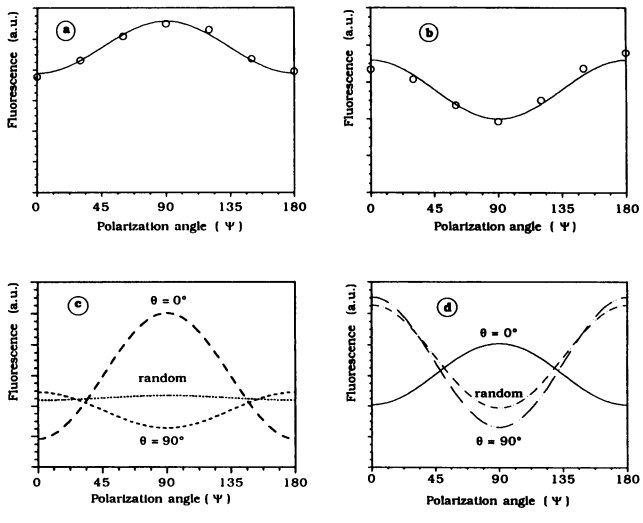

FIGURE 7 Fluorescence signal of porphyrin cytochrome *c* as a function of the polarization of the incident light beam. (*a*), (*b*) Experimental points and fitted curves ($F(\Psi) = A + B \cos^2 \Psi$) for porphyrin cytochrome *c* adsorbed on glass and on ITO, respectively. The concentration protein in solution was 10 mg/l; 10-mM phosphate buffer pH 7. (*c*), (*d*) Calculated curves for the adsorption on glass and on ITO, respectively, for a random distribution and sharp distributions with orientation angle $\theta = 0°$, 90°.

DISCUSSION

In the underlying theory for the TIRF orientation measurements several assumptions have been made (Bos and Kleijn, 1994). It is appropriate to establish whether these assumptions are justified for the systems under investigation.

First, it was assumed that the fluorescence lifetime of the molecules is much shorter than their rotational correlation time. The fluorescence lifetimes of H_2TMPyP and porphyrin cytochrome *c* in aqueous environment are 6.0 and 6.5 ns, respectively (Kalyanasundaram, 1984; Vanderkooi et al., 1976). The rotation correlation time of cytochrome in solution is ~5 ns (Vos et al., 1987). Generally, proteins form numerous contacts with a sorbent surface upon adsorption. Therefore, it is expected that the rotation correlation time in the adsorbed state will have a high value. Vanderkooi and Erecinska (1975) found a value of 200 ns for cytochrome *c* molecules bound to phospholipid layers. In the case of adsorption on a solid surface we expect even higher values, because the binding of proteins to such surfaces has an irreversible character (Norde and Haynes, 1994). For a small molecule such as H_2TMPyP the rotation correlation time in solution is of the order of 0.5 ns. Of course, in this case binding to a surface also limits the rotational mobility of the

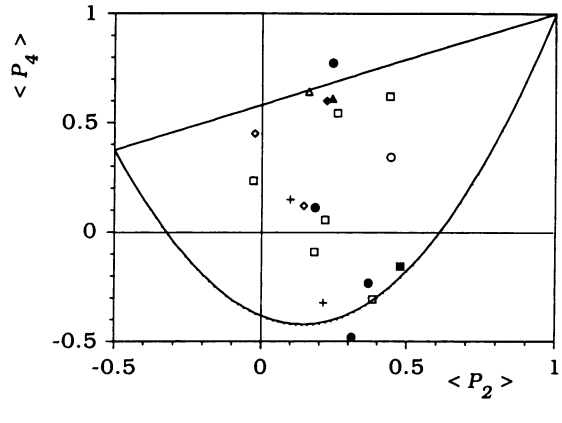

FIGURE 8 Order parameters for porphyrin cytochrome *c* adsorbed on glass and on ITO from 10 mM phosphate buffer pH 7. The physical boundaries of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are indicated. Open symbols: adsorption on glass from ○, 1-mg/l: □, 10-mg/l: △, 25-mg/l: ◇, 70-mg/l, and +, 100-mg/l protein solutions. Closed symbols: adsorption on ITO: ●, equilibrium potential; ■, −400 mV applied after adsorption at the equilibrium potential: ▲, +500 mV applied during adsorption; and ◆, +800 mV applied during adsorption; protein concentration 10 mg/l.

molecule. In the literature we did not find any data concerning the rotation correlation time of simple porphyrin molecules in the adsorbed state. Therefore, we performed orientation measurements on H_2TMPyP adsorbed on glass as a function of the viscosity of the solution phase (Bos, 1994). If the rotation correlation time in the adsorbed state were of the same order as the fluorescence time, a systematic effect on the fluorescence curves should be observed, i.e., the difference between the parallel and perpendicular polarized components would increase with increasing viscosity (Bos and Kleijn, 1994). Such an effect has not been found. Therefore, it is concluded that this first assumption is correct for both cytochrome *c* and H_2TMPyP.

A second basic assumption is that energy transfer between the adsorbed molecules is negligible. Inasmuch as for both H_2TMPyP and porphyrin cytochrome *c* the overlap between the absorption and emission spectra is poor (see Figs. 1 and 2), it is concluded that energy transfer is not interfering with the orientation measurements.

Finally, the assumption of isotropy in the *xy* plane, i.e., the interface, seems to be justified for the sorbent surfaces used. It is imaginable, in particular for the H_2TMPyP molecules, that there is some local ordening in the orientation angle ϕ (porphyrin rings of neighboring molecules are parallel), but the area of the interface to which the derived order parameters refer is so large (1 mm^2) that on average the orientation in ϕ can be assumed to be random.

The fact that for H_2TMPyP all, and for porphyrin cytochrome *c* almost all ($\langle P_2\rangle$;$\langle P_4\rangle$), combinations are found to be within their physical boundaries inspires confidence in the theoretical model and the values used for the various parameters. After all, these boundaries simply follow from the definitions of $\langle P_2\rangle$ and $\langle P_4\rangle$ and are model independent. Deviations between the measured values of $F_{\parallel}(0°)$, $F_{\perp}(0°)$, $F_{\parallel}(90°)$, and $F_{\perp}(90°)$ and their values predicted from the fitted order parameters are in the range of 2–15% for both H_2TMPyP and porphyrin cytochrome *c*. This is remarkable, because the observed scatter in the $\langle P_2\rangle$ and $\langle P_4\rangle$ data is more severe for porphyrin cytochrome *c* than for H_2TMPyP (see Figs. 5 and 7). Furthermore, the signal-to-noise ratio in $F(\Psi)$ is lower for the porphyrin cytochrome *c* adsorption layers, simply because the surface concentration of porphyrin groups is much lower.

A way to improve the signal-to-noise ratio is to measure $F(\Psi)$ over a longer time period. Unfortunately, for adsorbed porphyrin cytochrome *c* molecules this leads to changes in the fluorescence spectrum. Under conditions of continuous irradiation Fraaije et al. (1990) observed changes in the fluorescence spectrum of these molecules adsorbed on quartz, pointing to a change in the structure of the protein molecule resulting in exposure of the porphyrin ring to the solvent. Similar spectral changes were found here, although to a much lesser extent.

The difference between the orientation distributions for H_2TMPyP adsorbed from solutions with porphyrin concentrations of 1 and 10–100 mg/l can be related to the degree of surface coverage. The adsorption isotherm of H_2TMPyP on silica in 10-mM phosphate buffer pH 7 (Bos, 1994) shows that at 1 mg/l the adsorbed amount is approximately 0.8 mg/m^2, corresponding to an area of 2.5 nm^2 per molecule. At 10 and 100 mg/l these values are ~1.5 mg/m^2, corresponding to 1.3 nm^2 per molecule, and ~1.9 mg/m^2, corresponding to 1.1 nm^2 per molecule, respectively. Considering the size of the H_2TMPyP molecule (largest cross section ~2 nm^2), at 1 mg/l there is enough room left at the surface for the molecules to adopt any orientation angle θ without being (sterically) hindered by neighboring molecules. This is not the case at the higher degrees of occupancy. Therefore, the higher degree of ordering at 10 and 100 mg/l is probably due to intermolecular lateral interactions in the adsorption layer. The observed effect of porphyrin degrees of occupancy on the orientation distribution suggests that adsorption does not occur in clusters; otherwise also at low surface coverages this ordening by intermolecular interactions would occur.

For adsorption of porphyrin cytochrome *c* from 10-mM phosphate buffer pH 7 the adsorbed amounts are approximately 3 and 4 mg/m^2 for protein concentrations in solutions of 1 and 10 mg/l, respectively (Bos, 1994); the adsorption plateau amounts to 6 mg/m^2, which is reached at a solution concentration of 100 mg/l. Although the scatter in the obtained order parameters of the orientation distribution of porphyrin cytochrome *c* is too large to permit us to draw definite conclusions, from the fact that the total fluorescence curve $F(\Psi)$ does not significantly change with the surface coverage it appears that this factor does not have a decisive effect on the orientation of the adsorbed molecules. This might be explained by the fact that the cytochrome *c* molecule is almost spherical (Dickerson and Timkovich, 1975), so a decrease in available surface area per adsorbing molecule is not likely to result into a change in the average orientation. It should be noted, however, that most probably at the lowest solution concentration (1 mg/l) the surface is already completely covered with protein molecules and at the higher solution concentrations adsorption takes place in a second layer. (The adsorbed amount for a complete monolayer of cytochrome *c* molecules is estimated from their size (diameter 3 nm) and molecular weight (12200 g/mole) to be 2.5–3 mg/m^2.) Therefore, from our experiments we cannot rule out that below monolayer coverage the orientation of the molecules does vary with the adsorbed amount.

From the electrical double-layer theory it has been estimated that the electric field strength near the ITO surface varies with ~3 × 10^7 V/m per 100-mV change in the potential applied. At an imposed potential of ~350 mV versus the Ag/AgCl reference electrode, the potential difference between the ITO/solution interface and the bulk solution is zero (Bos et al., 1994). Thus, by varying the interfacial potentials between −400 and +800 mV versus Ag/AgCl, the absolute value of the electric field strength near the interface varies between 0 and 2 × 10^8 V/m, and the energy difference per molecule cytochrome *c* between the parallel and antiparallel orientation of its dipole in the field of the interface varies between 0 and 60 kT. Therefore, if electrostatic interactions were determining for the orientation in the adsorbed state, an

effect of the interfacial potential should be observable. However, such an effect is not observed in the $F(\Psi)$ curves. The direction of the electrical dipole moment of horse heart cytochrome *c* at pH 7 is known (Koppenol and Margoliash, 1982). The angle between this dipole moment and the plane of the haem group is 33° at this pH. If this also applies to the adsorbed porphyrin cytochrome *c* molecule, a perfect alignment of the electrical dipole along the electric field of the sorbent surface would yield values for $\langle P_2 \rangle$ and $\langle P_4 \rangle$ of −0.055 and −0.352. None of the experimentally obtained combinations of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ is close to these values. Therefore, we are strongly inclined to conclude that the imposed electrical potential does not affect the orientation distribution. This is further corroborated by reflectometer experiments conducted in our laboratory (Bos et al., 1994; Bos, 1994) in which the adsorption behavior of various proteins, including native and porphyrin cytochrome *c*, on ITO were examined as a function of an externally applied interfacial potential; for all proteins studied, the effect of this potential was found to be surprisingly small. Apparently, other factors than global electrostatic and dipole interactions, e.g., hydrophobic interactions and structural rearrangements, are dominating the adsorption process. An additional cause for the relative unimportant role of the applied potential might be that protein molecules can adjust their charge when approaching a surface and that coadsorption of small ions from solution occurs. The interaction between porphyrin cytochrome *c* and the ITO surface is apparently very different from that between the native protein and its physiological redox partners (see the introductory section).

A final important point of discussion, and directly related to the above, is of course the structural stability of the porphyrin cytochrome *c* molecule. Strong structural changes of the protein molecule upon adsorption would make it impossible to draw conclusions on the orientation of the adsorbed molecule from the orientation of the porphyrin group. The structural stability of native cytochrome *c* in solution is high. No structural changes take place between pH 3 and pH 12 (Dickerson and Timkovich, 1975; Timkovich, 1979). From studies performed in our laboratory (Bos, 1994) it was found that the adsorption behavior of cytochrome *c* resembles that of other structure-stable proteins such as lysozyme and ribonuclease (Norde, 1992). Therefore, it is likely that cytochrome *c* retains its structure when it adsorbs on hydrophylic surfaces. This is corroborated by the finding that in the adsorbed state cytochrome *c* still has, within experimental error, its crystallographically determined dimensions (Reynaud et al., 1986). However, it is much less clear whether the porphyrin derivative of cytochrome *c* retains its conformation during the adsorption process. On the one hand, its adsorption behavior is in many aspects the same as that of the native protein, and from competition and displacement measurements it is found that the two forms of the protein have the same affinity for the glass surface (Bos, 1994). On the other hand, porphyrin cytochrome *c* is more sensitive to heat and guanidine-HCl treatments than native cytochrome *c* (Vanderkooi and Erecinska, 1975), and, as stated above, the observed changes in the fluorescence spectrum suggest that under influence of light conformational changes at the interface occur. Anyway, the $F(\Psi)$ curves depicted in Fig. 7 (*a*) and (*b*) point to a certain degree of order in the adsorption layer; the orientation of the porphyrin groups is not random. Apparently, this order results from other factors than electrostatic interactions or the degree of surface coverage.

CONCLUSION

We have demonstrated that TIRF can be a useful tool in examining the orientation of fluorescent or fluorescent-labeled molecules at optically transparent surfaces. The results obtained here for adsorbed H_2TMPyP and porphyrin cytochrome *c* are encouraging, although for porphyrin cytochrome *c* the scatter in the obtained order parameters is substantial. This, together with the suspicion that porphyrin cytochrome *c* changes its conformation upon adsorption, makes it difficult to draw definite conclusions with respect to its orientation in the adsorbed state.

Conformational changes are a general problem encountered in the determination of the orientation of protein molecules in adsorption layers (Norde, 1992). A more specific problem in the application of TIRF to adsorbed proteins is the need for a fluorescent group. If the molecules do not carry a fluorescent group, one might consider labeling them; prerequisite is that the fluorescent label be fixed in the molecule with a known orientation. However, because the native structure of a protein molecule in general is only marginally stable from a thermodynamic point of view (Norde, 1992), introduction of a label might influence the structure or structural stability of the protein molecule and, hence, influence its adsorption behavior. Another possibility is to make use of the aromatic amino acids tryptophan and tyrosine. These amino acids have a fixed place in the structure. However, their excitation wavelength is in the UV, which sets high demands on the experimental setup, and protein molecules might easily be damaged by the UV light. Furthermore, the presence of more than one of these amino acids might cause energy transfer from one amino acid to the other, which will severely complicate efforts to extract information on the orientation of the molecules.

Meanwhile, the TIRF method for determining the orientation of adsorbed fluorophores has already been used in a study concerning the development of "organic" solar cells (Wienke et al., 1994). In this study porphyrin molecules were used as sensitizers to generate charge carriers in a semiconducting surface. The orientation of these porphyrins is considered to be a prominent factor in determining the efficiency of the system. In our own institution the method is now used to investigate the order in Langmuir–Blodgett layers of phospholipids, which stand model for biological membranes.

REFERENCES

Bos, M. A. 1994. TIRF and its application to protein adsorption. Electrostatics and orientation, Ph.D. dissertation. Wageningen Agricultural University, The Netherlands.

Bos, M. A., and J. M. Kleijn. 1994. Determination of the orientation distribution of adsorbed fluorophores. I. Theory. *Biophys. J.* In press.

Bos, M. A., Z. Shervani, A. C. I. Anusiem, M. Giesbers, W. Norde, and J. M. Kleijn. 1994. Influence of the electrical potential of the interface on the adsorption of proteins. *Coll. Surf. B.* 3:91–100.

Burghardt, T. P., and N. L. Thompson. 1984. Effect of planar dielectric interfaces on fluorescence emission and detection. Evanescent excitation with high-aperture collection. *Biophys. J.* 46:729–737.

Dickerson R. E., and R. Timkovich. 1975. Cytochromes *c*. *In* The Enzymes. Vol. XI, Oxidation-Reduction, part A. P. D. Boyer, editor. Academic Press, New York. 397–547.

Fisher, W. R., H. Taniuchi, and C. B. Anfinsen. 1973. On the role of heme in the formation of the structure of cytochrome *c*. *J. Biol. Chem.* 248: 3188–3195.

Flatmark T., and A. B. Robinson. 1968. *In* Structure and Function of Cytochromes. K. Okunuki, M. D. Kamen, and I. Sekuzu, editors. University Park Press, Baltimore. 383–387.

Fraaije, J. G. E. M., J. M. Kleijn, M. Van der Graaf, and J. C. Dijt. 1990. Orientation of adsorbed cytochrome c as a function of the electrical potential of the interface studied by total internal reflection fluorescence. *Biophys. J.* 57:965–975.

Hansen, W. N. 1968. Electric fields produced by the propagation of plane coherent electromagnetic radiation in a stratified medium. *J. Opt. Soc. Am.* 58:380–390.

Harrick, N. J. 1967. Internal Reflection Spectroscopy. Wiley/Interscience, New York.

Ivarsson, B. A., P.-O. Hegg, K. I. Lundström, and U. Jönsson. 1985. Adsorption of proteins on metal surfaces studied by ellipsometric and capacitance measurements. *Coll. Surf.* 13:169–192.

Kalyanasundaram, K., and M. Neumann-Spallart. 1982. Photophysical and redox properties of water-soluble porphyrins in aqueous media. *J. Phys. Chem.* 86:5136–5169.

Kalyanasundaram, K. 1984. Photochemistry of water-soluble porphyrins: comparative study of isomeric tetrapyridyl- and tetrakis(*N*-methylpyridiniumyl)porphyrins. *Inorg. Chem.* 23:2453–2459.

Koppenol, W. H., and E. Margoliash. 1982. The asymmetric distribution of charges on the surface of horse cytochrome *c*. *J. Biol. Chem.* 257:4426–4437.

Norde, W. 1992. The behavior of proteins at interfaces, with special attention to the role of the structure stability of the protein molecule. *Clin. Mater.* 11:85–91.

Norde, W., and C. A. Haynes. 1994. Reversibility and the mechanism of protein adsorption. *ACS Symp. Ser.* In press.

Reynaud, J. A., I. Tavernier, L. T. Yu, and J. M. Cochet. 1986. The adsorption of RNase A, BSA and cytochrome *c* at the graphite powder/liquid interface using in parallel the adsorption isotherm plot and linear sweep voltammetry on graphite paste electrode. *Bioelectrochem. Bioenerg.* 15: 103–112.

Timkovich, R. 1979. Cytochrome *c*: the architecture of a protein-porphyrin complex. *In* The Porphyrins. Vol. 7. Biochemistry, part B. D. Dolphin, editor. Academic Press, New York. 241–294.

Vanderkooi, J. M., and M. Erecinska. 1975. Cytochrome c interactions with membranes. Absorption and emission spectra and binding characteristics of iron-free cytochrome *c*. *Eur. J. Biochem.* 60:199–207.

Vanderkooi, J. M., F. Adar, and M. Erecinska. 1976. Metallocytochromes *c*: characterization of electronic absorption and emission spectra of Sn^{4+} and Zn^{2+} cytochromes *c*. *Eur. J. Biochem.* 64:381–387.

Vos, K., C. Laane, S. R. Weijers, A. van Hoek, C. Veeger, and A. W. J. Visser. 1987. Time-resolved fluorescence and circular dichroism of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* incorporated in reversed micelles. *Eur. J. Biochem.* 169:259–268.

Wienke, J., F. Kleima, R. B. M. Koehorst, and T. J. Schaafsma. 1994. Orientation of various free-base methylpyridinium porphyrins. *In* Proc. 12th European Photovoltaic Solar Energy Conference. R. Hill, W. Palz and P. Helm, editors. H. S. Steven Associates, Bedford, UK. 5.64–5.67.