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Orientational Distributions of the Di- α -helical Synthetic Peptide ZnPPIX-BBC16 in Langmuir Monolayers by X-ray Reflectivity and Polarized Epifluorescence

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The vectorial orientation of the peptide ZnPPIX-BBC16 in a Langmuir monolayer was studied by means of X-ray reflectivity and polarized epifluorescence. ZnPPIX-BBC16 consists of two α -helical 31-mers dimerized via a disulfide bridge between two N-terminal cysteines (α -S-S- α), so that two bis-his metalloporphyrin binding sites between positions 10,10' and 24,24' are formed. Zn(II) protoporphyrin IX was bound to the position 24. To enhance stability at the interface, a palmitoyl (C16) chain was bonded to each N-terminal cysteine. X-ray reflectivity measurements make it possible to infer the orientation of the α -helices by determining the electron density profile of the monolayer. Polarized epifluorescence provides the orientation distribution of the porphyrin with respect to the monolayer plane. Both X-ray and fluorescence measurements show that at low surface pressure ($\pi = 5$ mN/m), the peptide α -helices lie in the plane of the water surface with a narrow orientational distribution of the porphyrin. At high pressures ($\pi > 30$ mN/m), the peptide α -helices are perpendicular to the water surface and the distribution is wider. At intermediate pressures the peptide α -helices in the monolayer undergo a transition from the parallel to perpendicular orientation with almost a complete loss of the orientational order of the porphyrin.

1. Introduction

Monolayer films of oriented natural or synthetic proteins containing metalloporphyrin, a major effector of biological oxidation–reduction reactions, are very promising for fundamental biophysical studies and biotechnology applications. One of the well-established techniques for fabricating ordered monolayers on solid supports is the Langmuir–Blodgett (LB) method. The structure of a LB film is determined to a substantial degree by that of the precursor Langmuir monolayer formed at the air–water interface. In the case of protein Langmuir films, the orientation of the molecules in the monolayer depends to a great extent on external parameters such as surface pressure. It has been shown that reorientation due to changes in surface pressure occurs in monolayers composed of very different proteins, such as immunoglobulin G,¹ cytochrome p450ssc,² photosynthetic reaction centers,³ and the synthetic peptide BBC16.⁴ Thus, the LB technique makes it possible to control molecular orientation via surface pressure, which can be very important for making functional films. Knowing the orientation of the molecules in the precursor Langmuir monolayer at the air–water interface is therefore very important for creating LB films with desired structure. A very powerful technique for

obtaining structural information about a monolayer is X-ray reflectivity (XR), which can determine the electron density profile of a monolayer.⁵ Another promising technique for studying molecular orientation is the recently introduced polarized epifluorescence (PEF) technique.⁶ In this technique, the orientation of the fluorophore (Zn-porphyrin in this case) can be determined by measuring the polarization of the fluorescence excited by an electric field directed normal to and along the film surface. The main advantage of fluorescence measurements over other linear optical techniques is that fluorescence, a “two-photon” process, makes it possible to determine not only the mean orientation angle of the porphyrin with respect to the monolayer plane but also the width of the orientational distribution.^{6–8} This feature is of particular importance since it gives a measure for the degree of the orientational order of the monolayer. Both XR and PEF can be applied to the study of monolayers at the air–water interface and provide complementary information on the orientation of the peptide itself and the functional group, respectively. Recently we have studied the Langmuir monolayers of apoBBC16 (i.e., BBC16 without metalloporphyrin bound).⁴ We showed that the peptide orientation changes with surface pressure. At low pressure ($\pi < 22$ mN/m) the peptide is oriented with its dihelices lying in the plane of the monolayer, whereas at high pressure ($\pi > 30$ mN/m) the peptide's dihelices are perpendicular to the surface. We were not able to infer any knowledge about the orientational order, since the absence of the suitable fluorophore did not permit the

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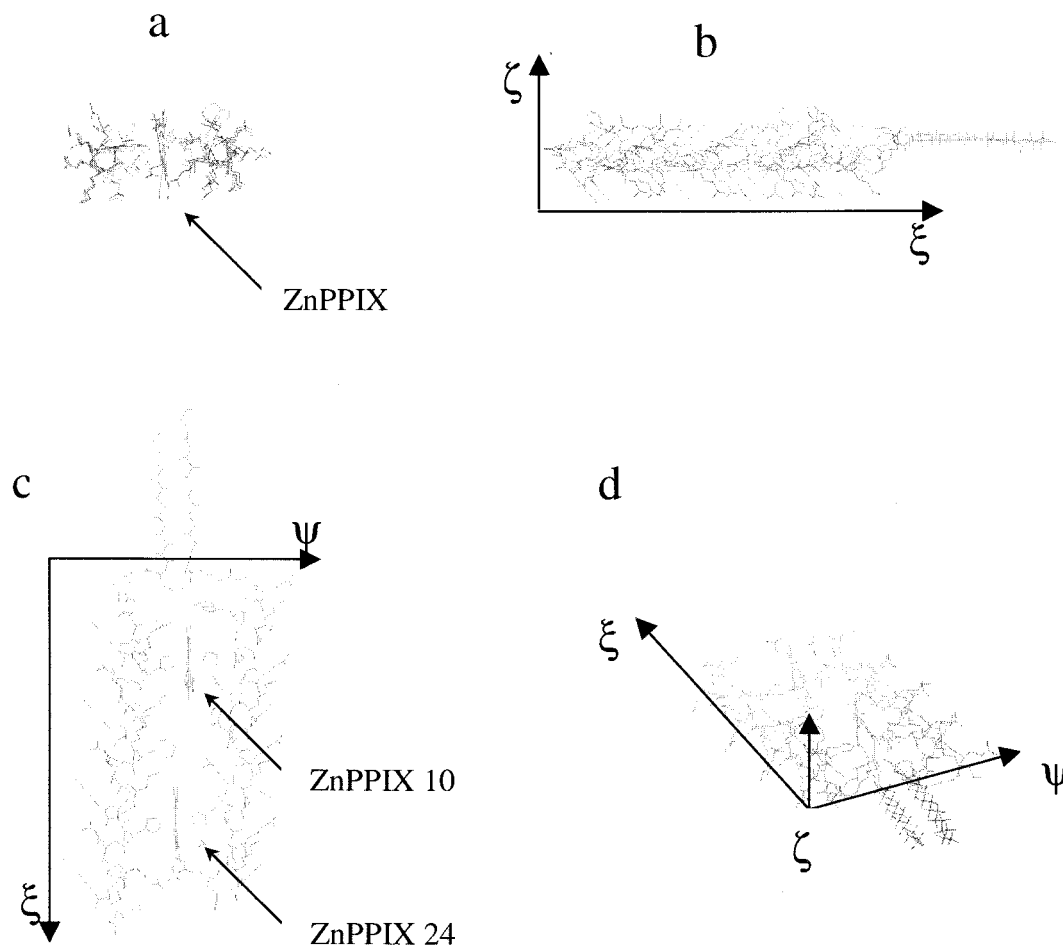


Figure 1. Schematic structure of ZnPPIX-BBC16: (a, b, c) mutually perpendicular projections; (d) isometric projection. ξ and ψ axes lie in the plane of the axes of the two α -helices; ξ is parallel and ψ is perpendicular to the axes of the two α -helices. The porphyrin plane is perpendicular to the ψ axis.

fluorescence measurements. In the present paper we study the monolayers of the porphyrin containing or holoform of the same peptide, ZnPPIX-BBC16. The structure of these two forms is very similar, so we expect similar results for the ZnPPIX-BBC16. Presence of the Zn-porphyrin in the peptide structure allows us to apply PEF.

The synthetic peptide was designed de novo as a model for intramolecular electron transfer. The synthetic unit is an α -helical 31-mer with a palmitoyl (C16) chain bonded to the N-terminal cysteine to increase the peptide's amphiphilicity. The units dimerize via a disulfide bridge between two cysteines (α -S-S- α) to form two bis-his metalloporphyrin binding sites between sequence positions 10,10' and 24,24'. The schematic peptide structure is shown in Figure 1. We define the peptide frame by the three axes ξ , ψ , and ζ . The approximate dimensions of the peptide group, without the hydrocarbon chains, are 50, 20, and 10 Å, respectively. We will call the (ξ, ψ) plane the peptide plane, referring to the largest, smallest, and medium dimensions as "length", "thickness", and "width" of the molecule. Pronounced differences in the length and thickness make it possible to infer the molecular orientation from the measurements of the monolayer electron density profile.⁴ The porphyrin plane is perpendicular to the ψ -axis. The metalloporphyrins shown occupy both binding sites. In solution, however, the metalloporphyrin affinity to the first site is low possibly due to interference from the palmitoyl chain, and it is possible to bind porphyrin in the 24,24' position only. However, upon subsequent spreading in a Langmuir monolayer, this interference should be removed resulting in the metal-

loporphyrin's redistribution to occupy both the 10,10' and 24,24' binding sites.

2. Materials and Methods

BBC16 was synthesized according to ref 9. Zn^{II}PPIX was purchased from Porphyrin Products, Inc. To bind Zn^{II}PPIX to BBC16, the equivalent of 1 porphyrin per 1 dihelix was titrated into a 0.1 mM BBC16 solution in 1 mM phosphate buffer with 10 mM NaCl, pH 8, from a 2.7 mM solution of Zn^{II}PPIX in DMSO in accordance with ref 10. The concentration of the peptide solution was determined spectroscopically by measuring the absorption at $\lambda = 280$ nm.^{10,11} The titration of Zn^{II}PPIX was monitored spectroscopically by observing the red shift of the Soret absorption band from 404 nm for free Zn^{II}PPIX to 427 nm, which corresponds to the metalloporphyrin in bis-his coordination. The resulting solution was used for spreading the Langmuir monolayer. To prepare the monolayer of ZnPPIX-BBC16, a technique described in ref 4 was used with the exception that in the present study we used only pure peptide monolayers without any addition of a phospholipid. X-ray reflectivity measurements were performed at beamline X22B of the National Synchrotron Light Source using a previously described Liquid Surface spectrometer⁴ with a small, custom-built Langmuir trough (Ames Lab, Ames, IA) mounted on the sample stage. XR data were analyzed using box refinement (BR), an iterative, model-independent procedure for recovering the electron density profile, $\rho(Z)$, of a monolayer

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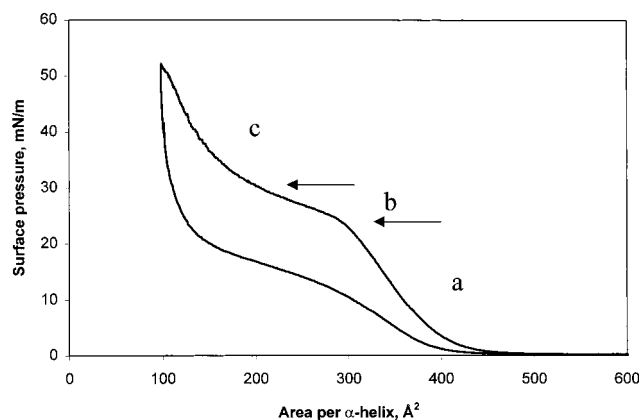


Figure 2. Compression–expansion isotherm of the monolayer of ZnPPIX-BBC16. See text for details.

from the data.^{12,13} For the details of the BR application to the Langmuir monolayer case see refs 4 and 14.

The polarized epifluorescence measurements were performed with a custom-built fluorimeter, described in detail elsewhere.⁶ The design makes it possible to acquire fluorescence intensity data directly from the air–water interface. The Langmuir trough used in epifluorescence measurements was a custom trough from Riegler & Kirstein GmbH. The wavelength of the excitation beam was $\lambda = 514$ nm. The fluorescence was observed through a 570 nm cutoff filter (Melles-Griot). To acquire the background signal for the fluorescence measurements, monolayers of apoBBC16 otherwise prepared identically were used. Acquisition time for each fluorescence intensity measurement was 5 s. Intensities were repeatedly acquired in the order I_{sx} , I_{px} , I_{py} , I_{sy} , I_{sx} , I_{py} , I_{px} , I_{sy} , etc. (indices p , s indicate the polarization of the excitation field and x , y indicate the polarization of the emitted field), to get sufficient statistics and to eliminate the influence of porphyrin photobleaching.⁷ The overall errors of the measured intensities due to the noise and photobleaching were less than 1%.

3. Results and Discussion

3.1. Compression Isotherms. Figure 2, an isotherm collected from a Langmuir monolayer of the ZnPPIX-BBC16, shows how the surface pressure (π) varies as a function of the area of the monolayer. Compression and expansion show pronounced hysteresis. There are at least three regions with different compressibility that can be distinguished in the compression curve. The first one, denoted as “a” on the figure, begins with the surface pressure onset at about 400 Å^2 per α -helix and extends to $\pi \approx 22 \text{ mN/m}$. This region is characterized by low compressibility. The second one (“b”), with much higher compressibility, begins at the end of region a and ends at $\pi \approx 30 \text{ mN/m}$, where the curve exhibits the sharp kink and the compressibility becomes low again. The third region (“c”) begins at this kink. The value for the limiting area per α -helix for the third region is 100 Å^2 , which is about the smallest cross section of the α -helix.

3.2. X-ray Reflectivity. Pronounced variations of the monolayer compressibility suggest some structural changes. To get insight in the peptide orientation, we used X-ray reflectivity measurements in each of these three regions. Figure 3 shows X-ray reflectivity data, normalized by the Fresnel reflectivity for an infinitely sharp water–helium interface, taken at five surface pressure values, namely, 5 mN/m (region a), 22 and 27 mN/m (region b), and 30 and 34 mN/m (region c). Experimental data are shown by markers, calculated results from box refinement

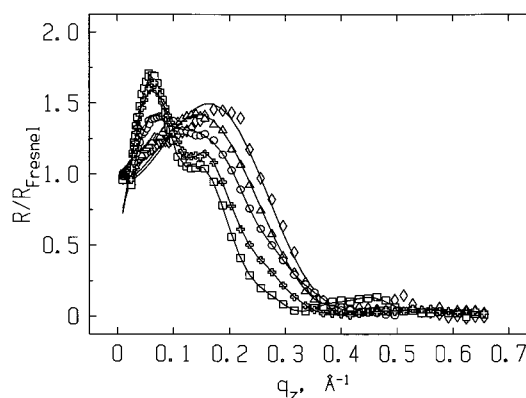


Figure 3. Modulus of the X-ray reflectivity divided by the Fresnel reflectivity of pure water vs surface pressure of the Langmuir monolayer of ZnPPIXBBC16: diamonds, $\pi = 5 \text{ mN/m}$; triangles, $\pi = 22 \text{ mN/m}$; circles, $\pi = 27 \text{ mN/m}$; crosses, $\pi = 30 \text{ mN/m}$; squares, $\pi = 34 \text{ mN/m}$. Experimental data are shown by markers; lines show the calculation for the electron density profiles determined via the box refinement procedure.

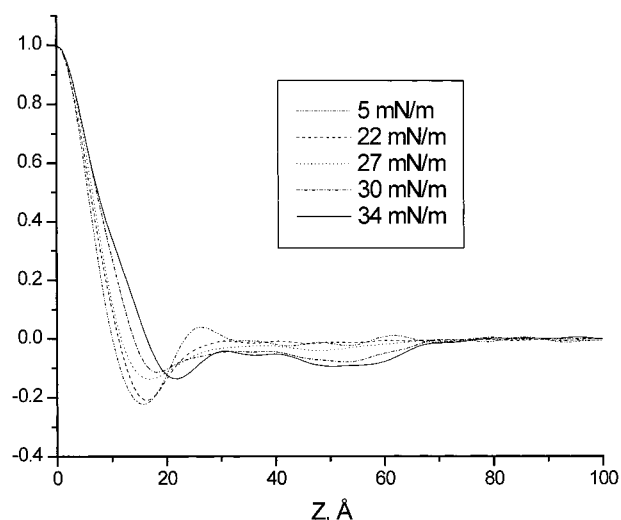


Figure 4. Autocorrelation function for the gradient of the electron density profile of the Langmuir monolayer of ZnPPIX-BBC16 vs surface pressure.

(see below) are shown by the continuous curves. One can see systematic changes in the reflectivity with the surface pressure. The curves for the regions a and b have one broad maximum over the range of momentum transfer shown, whereas those for the region c have several maxima much narrower in width over the same range. Throughout regions a–c, the overall normalized X-ray reflectivity shifts toward lower values of momentum transfer and the narrower maxima become more pronounced as π increases suggesting that the monolayer thickness increases. The thickness can be determined directly by calculating the autocorrelation of the gradient of the monolayer electron density profile. The range in which the autocorrelation function has nonzero values yields the maximum extent of the monolayer structure in the direction perpendicular to the surface (Z -direction). One of the main advantages of the autocorrelation function is that it is computed directly from the experimental normalized reflectivity data without imposing any a priori conditions. The results of the computation are shown in Figure 4. The most evident difference is between the curves corresponding to regions a and c. The autocorrelation functions from the low- π region a decay to zero by $Z = 20 \text{ Å}$ while those from the high- π region c extend to about 60 Å and include a second minimum in the vicinity of 55 Å that is absent in the

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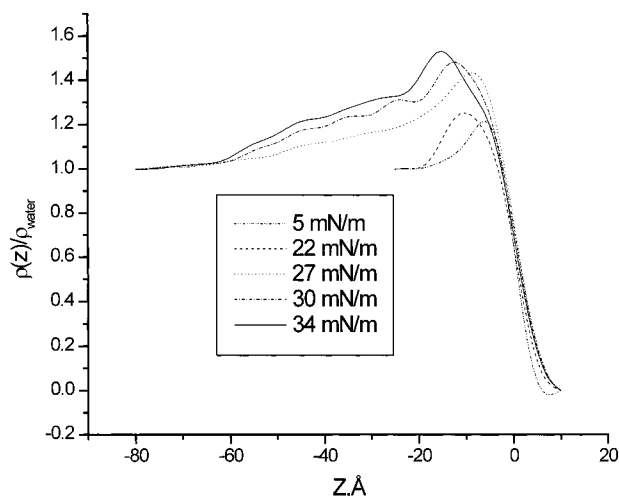


Figure 5. Electron density profile of the Langmuir monolayer of ZnPPIX-BBC16 vs surface pressure obtained via a box refinement procedure. Zero of the Z axis corresponds to the air–water interface; the positive direction of the axis is toward air. Electron density is normalized to that of the water, so that the value of 1 corresponds to the subphase, and the value of 0 to the air.

low- π functions. The curve from region b at $\pi = 27$ mN/m has a weak second minimum and may represent an intermediate case. Looking at the individual curves from regions a and b more closely, we see that the location of the first minimum in the autocorrelation function varies with π from $Z = 14$ Å at $\pi = 5$ mN/m to $Z = 17$ Å at $\pi = 27$ mN/m. In contrast, the location of the second minimum in the functions for region c shows no noticeable dependence on surface pressure.

The change in monolayer thickness with surface pressure is clearly seen in the electron density profiles obtained via box refinement from the XR data (Figure 5). Helium ($\rho \approx 0$) fills the positive half-space while the semi-infinite subphase ($\rho = 1$) extends to the left. In our calculation we normalized electron density $\rho(Z)$ to that of the water, $\rho_w = 0.333 \text{ e}^-/\text{\AA}^2$. The region with density $\rho > 1$ is the peptide monolayer. At lower pressures the monolayer thickness systematically increases from 10 Å for $\pi = 5$ mN/m to 15 Å for $\pi = 22$ mN/m. At higher pressure the thickness increases drastically up to 50 Å. Normalized reflectivity curves, calculated for the resulting electron density profiles, are shown in Figure 3 by the continuous curves and agree well with the experimental data.

3.3. Polarized Epifluorescence. To get more information on the peptide orientation in the monolayer, we used polarized epifluorescence measurements. PEF and XR measurements were not performed at exactly the same surface pressures but covered overlapping ranges. PEF measurements were done at $\pi = 5, 22, 32$, and 40 mN/m. We define a tilt angle θ as the angle between the normal of the porphyrin ring and the normal of the monolayer plane. Assuming that the tilt angle obeys a Gaussian distribution

$$P(\theta) = \frac{\exp(-(\theta - \theta_m)^2/2\sigma^2)}{\int_0^\pi \exp(-(\theta - \theta_m)^2/2\sigma^2) d\theta}$$

we can apply PEF to measure the mean tilt angle, θ_m , and the width of the distribution, σ . The distribution parameters obtained are given in Table 1. The quality of the fits was sufficiently good that the discrepancies between the measured and calculated intensities were less than the

Table 1. Distribution Parameters for the Langmuir Monolayer of ZnPPIX-BBC16 vs Surface Pressure

surface pressure, mN/m	mean tilt angle, deg	distribution width, deg
5	90	8
22	65	70
32	67	50
40	74	35

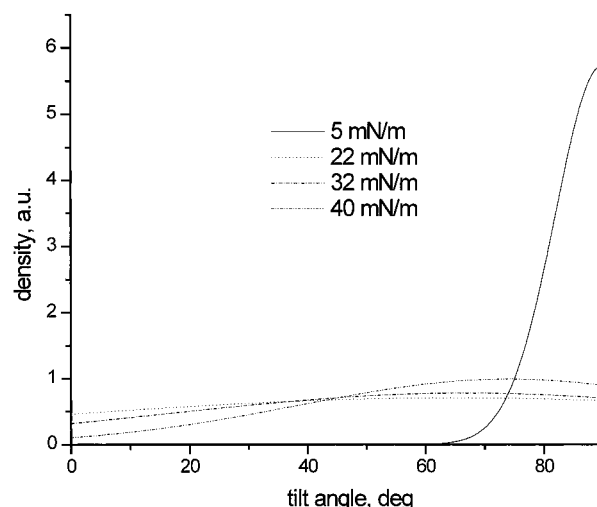


Figure 6. Porphyrin orientation distribution for Langmuir monolayers of ZnPPIX-BBC16 vs surface pressure as determined from the polarized epifluorescence measurements.

experimental errors. The corresponding calculated Gaussian distribution curves are shown in Figure 6. At the lowest pressure the planes of the porphyrins are oriented on average perpendicular to the monolayer plane with a high degree of orientational ordering, i.e., a relatively narrow distribution width. For $\pi = 22$ mN/m the porphyrin orientation changes dramatically: the mean tilt angle drops to the value of 65° and the distribution becomes very broad. With further increasing pressure, the tilt angle shifts back toward 90°, and the distribution becomes narrower. At the maximum surface pressure the average porphyrin orientation is close to perpendicular again. It was shown⁶ that the mean tilt and the distribution width as determined by polarized fluorescence are highly interrelated, and the accuracy of the orientation distribution determination is given by some range of (θ_m, σ) values which comply with the fluorescence intensities. These regions of allowed distribution parameters are shown in Figure 7. At the lowest surface pressure the mean tilt and width are confined to a small region with $75^\circ < \theta_m < 90^\circ$ and $0^\circ < \sigma < 18^\circ$. For $\pi = 22$ mN/m the region of the allowed θ_m and σ becomes very broad in both the tilt and width with $25^\circ < \theta_m < 90^\circ$ and $0^\circ < \sigma < 90^\circ$. We note here that for this intermediate pressure, the determined Gaussian distribution is so broad that the relative number of porphyrins within the vicinity of the mean tilt angle is only slightly greater than the numbers at the extremes of the distribution, as shown in Figure 6. It is possible that for such broad distributions, a bimodal distribution might be a better description of the tilt angle distribution, but the number of parameters required for this determination is beyond the current scope of the technique employed. For $\pi = 32$ and 40 mN/m the region is much more confined than for $\pi = 22$ mN/m. With an increase of surface pressure from 32 to 40 mN/m the allowed region shifts toward higher values of θ_m and lower values of σ . Although the range of the allowed θ_m values remains rather broad, the allowed mean tilt angles for the

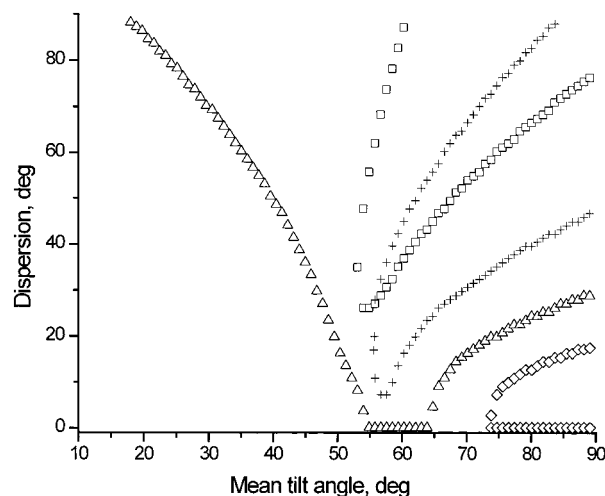


Figure 7. Uncertainty of the (θ_m, σ) determination for Langmuir monolayers of ZnPPIX-BBC16: $\pi = 5$ mN/m, diamonds; $\pi = 22$ mN/m, triangles; $\pi = 32$ mN/m, squares; $\pi = 40$ mN/m, crosses. For the surface pressure $\pi = 5$ mN/m the regions of possible (θ_m, σ) values are limited by the shown curve and the line $\theta_m = 90^\circ$, for the higher surface pressure the regions are limited by the respective curves and the lines $\theta_m = 90^\circ$ and $\sigma = 90^\circ$. Uncertainty is due to errors in the measurement of the fluorescence intensities.

porphyrins must be greater than 50° , since the values of $\theta_m < 50^\circ$ do not agree with the fluorescence measurements for either of the two higher pressures.

4. Discussion

Both X-ray reflectivity and polarized fluorescence measurements provide strong evidence that the peptide dihelices lie in the monolayer plane at the lowest pressure studied (see Figure 8a). The electron density profile shows that the monolayer thickness is about 10 Å. This value coincides with the thickness of only one α -helix while the dihelix would be double that value. The porphyrin rings are perpendicular to the surface in this case, in agreement with the fluorescence measurements.

In the range c the monolayer thickness is 50 Å. This value coincides with the peptide length and can be achieved by perpendicular orientation of the peptide dihelices (Figure 8b). Note that the thickness is constant in the range of surface pressure $30 < \pi < 34$ mN/m, which allows us to rule out any kind of the peptide multilayer formation. The perpendicular orientation is supported by the surface pressure isotherm, because the limiting area in this pressure range coincides with the smallest cross section of the α -helix. The porphyrin tilt angle is again about 90° for the perpendicularly oriented peptide, which agrees with (or at least does not contradict) the results of the fluorescence measurements. The behavior of the monolayer of the ZnPPIX-BBC16 is similar to that of apoBBC16, studied previously by X-ray reflectivity.⁴ The latter undergoes a sharper transition from the parallel orientation to the perpendicular one.

In the range a, the monolayer thickness increases slightly with pressure. This increase could originate from the peptide tilting about either the ξ or ψ axis (defined in Figure 1). However, any rotation about the ψ -axis does not cause changes in the porphyrin tilt angle (Figure 8d). The porphyrin, being perpendicular to the rotation axis, remains in the same plane. In the case of rotation about the ξ axis (Figure 8c) the porphyrin tilt angle changes by the same amount. The increase of the thickness Δd is related to the tilt angle as $\Delta d = l \cos \theta_m$, where l is the

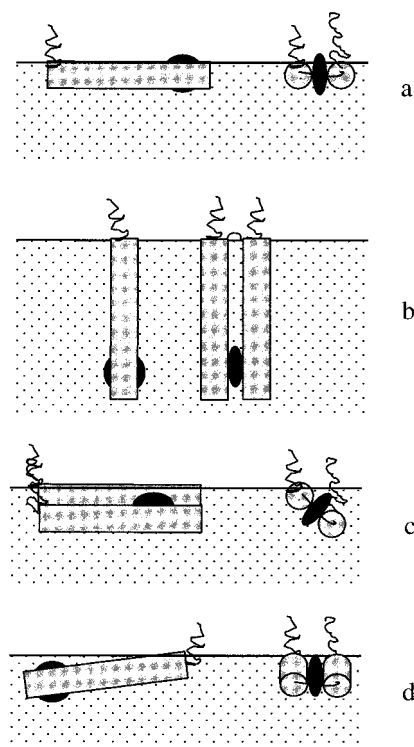


Figure 8. Scheme of the ZnPPIX orientation in the Langmuir monolayer: (a) low surface pressure, $\pi < 22$ mN/m; (b) high surface pressure, $\pi > 30$ mN/m; (c, d) intermediate surface pressure, $22 < \pi < 30$ mN/m.

peptide width. For $\Delta d = 5$ Å and $l = 20$ Å, we predict $\theta_m = 75^\circ$, which agrees reasonably well with the value of $\theta_m = 65^\circ$ obtained from the fluorescence measurements. The latter also show that the porphyrin orientation distribution is extremely wide in the region b, which means that the peptides are almost completely disordered with respect to the rotation around the ξ axis.

At $\pi = 27$ mN/m there appears to be a coexistence between two peptide orientations. The broad shoulder to the left of the peak in the electron density profile at this pressure does have a length that corresponds to peptide dihelices oriented perpendicular to the interface. However, the low density of the shoulder, especially as relative to that for the higher pressures, suggests that the fraction of molecules in the perpendicular orientation is small, and the majority of the monolayer is composed of peptide molecules whose dihelices remain oriented parallel to the interface.

To better understand the behavior of the peptide at the air–water interface, it is useful to consider the distribution of the hydrophobic/hydrophilic areas over the surface of the peptide molecule. The molecular structure of the dihelical peptide, obtained as a result of solution NMR technique¹⁵ is shown in Figure 9. The hydrophobic residues are marked by black. There is a significant amount of exposed hydrophobic residue, located mostly on one face of the molecule. Such a distribution should render some surface activity to the molecule.

The experimental observations and molecular structure allow us to propose the following model for the monolayer structure. In the surface pressure range a, the peptide dihelices lie in the plane of the surface. The hydrophobic face is directed away from the subphase, providing a high degree of the orientational order. The compressibility of

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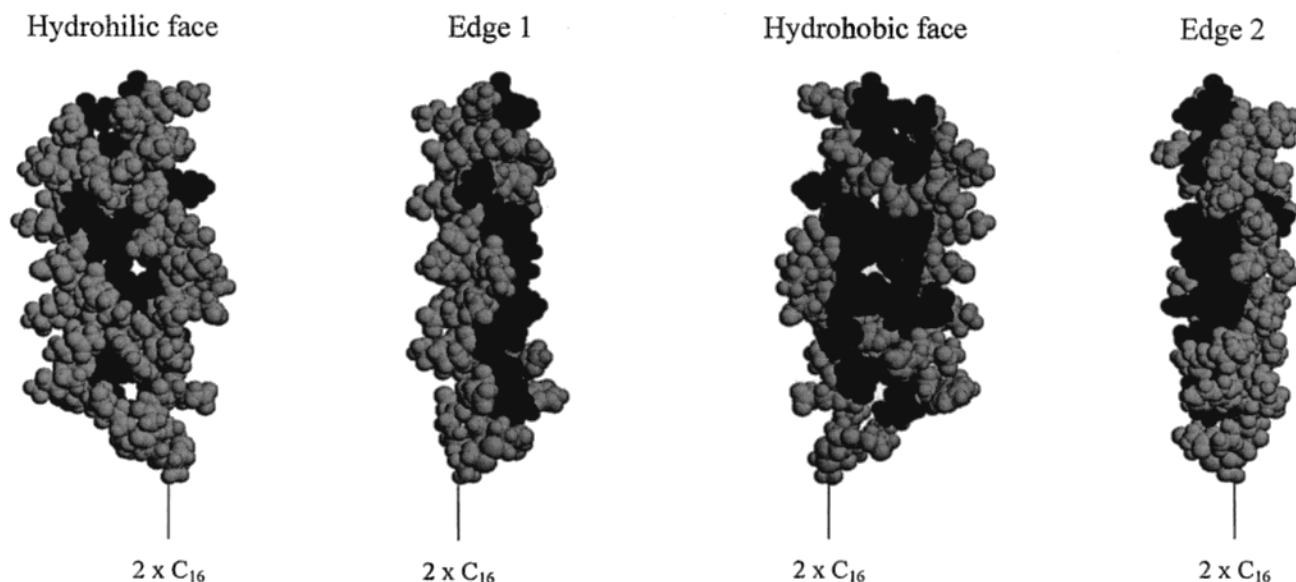


Figure 9. Distribution of hydrophilic/hydrophobic residues over the surface of BBC16. Hydrophobic residues (Ala, Val, Phe, Pro, Ile, Leu) are shown in black. The views show the molecule at four successive rotations about the ξ axis by 90° .

the monolayer being restricted by the molecular interactions is low. In the range b the molecules begin to rotate around the ξ axis, the monolayer thickness and compressibility increase, and the orientational order of the Zn-porphyrins decreases. The hydrophobic faces are no longer totally exposed to the air; they are forced into the subphase by the surface pressure. With further pressure increase the average time during which the hydrophobic faces contact the water increases (as does the number of the molecules with the immersed hydrophobic faces). Once a sufficient fraction of the molecules become oriented with their ψ axis perpendicular to the water surface, the possibility arises that neighboring immersed molecules can associate to form dimers in which the hydrophobic faces are apposed, and the external surface of the dimer is hydrophilic. Such dimers are much less surface active; they are anchored to the interface by the hydrocarbon chains only, and the peptide domains can go into the subphase without increasing the energy of the system. At this point the monolayer structure changes abruptly. The peptide dihelices take the perpendicular orientation, and the monolayer thickness increases to the value of 50 Å. The vectorial orientation is provided by the surface area limitation in this range, and the orientational order increases as the area per molecule decreases. The idea

that the change of the orientation is associated with the dimer formation is supported by the various experimental data showing that the dihelical peptides without the hydrocarbon chain forms dimers ("four-helix bundles") in solution.¹⁵

In conclusion we can say that the X-ray reflectivity and polarized epifluorescence data provide strong evidence of the reorientation of the ZnPPIX-BBC16 in the Langmuir monolayer. At lower surface pressure the peptide's dihelices lie in the plane of the surface with a high degree of orientational order. At high pressure they orient perpendicular to the interface but are less well ordered. In the intermediate region the molecules rotate around the dihelical long axis with almost complete loss of orientational order of the porphyrins. It seems plausible that the transition from the in-plane to the perpendicular orientation is accompanied by the formation of the dimers of the peptide, i.e., four-helix bundles.

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