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Determination of the amount of native structural bacteriorhodopsin in purple membrane Langmuir-Blodgett films by a spectroscopic surface denaturation quantifying technique

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Abstract

Purple membrane (PM) shows denaturation when spread over an air/water interface. We established a technique, which we call the spectroscopic surface denaturation quantifying (SSDQ) technique, that uses infrared linear dichroism to determine the amount of native structural bacteriorhodopsin (BR) in PM Langmuir-Blodgett (LB) films. Using the SSDQ technique we found that the conformational change after surface denaturation of BR was the same as that caused by ethanol treatment. By extrapolating the data of the amount of non-denatured BR molecules in PM LB films vs. the area of a single BR molecule on an air/water interface, we also found that the surface area of a single non-denatured BR molecule was 11.5 nm^2, which is consistent with that determined by high-resolution electron cryo-microscopy and electron diffraction (EMD). These results demonstrate that the SSDQ technique is effective in quantifying the amount of native structural BR in PM LB films. The SSDQ technique is also applicable to other types of protein consisting of α-helical conformation.

Keywords: Purple membrane; Bacteriorhodopsin; Langmuir-Blodgett technique; Fourier transform infrared spectroscopy; α-Helix

1. Introduction

Before proteins can be applied to bioelectronic devices such as two-dimensional (2D) image sensor with a photosensitive protein array, protein-handling quantitative analysis techniques must be developed for reconstituted or rearranged proteins. The Langmuir-Blodgett (LB) method has been the one most commonly used to obtain highly ordered reconstituted proteins and utilizes the protein's spontaneous orientation on an air/water interface. Purple membrane (PM) from *Halobacterium halobium* (*H. halobium*) consists of a single membrane protein, bacteriorhodopsin (BR), crystallized in the bilayer plane as a 2D hexagonal array [1,2]. This PM has been a good specimen for the LB method because it is exceptionally stable among proteins. BR consists of a single 26 866 Da polypeptide chain of 248 amino acids. It comprises 75% of the PM dry weight, with the

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remainder being lipids. The polypeptide chain is enfolded seven times into the PM bilayer, embedding ~80% of the amino acids within the hydrophobic environment of the membrane. The bilayer-spanning polypeptide segments are predominantly α-helical in their secondary structure. An oriented film of PM fragments has photovoltaic properties due to the light-driven proton pumping function of BR [3,4].

PM, even with its high stability, is known to denature when spread over an air/water interface. Furuno et al. [4] directly observed (using an SEM) the transfer of the PM interface film onto a silicon wafer. They concluded that the intermembrane region (i.e., the region excepting the region of randomly distributed PM fragments in an interface film) was occupied by either denatured PM or unfolded BR from the periphery of PM fragments at the air/water interface. For the direct observation of a PM interface film, an SEM is better than freeze-fracture electron microscopy [3] because the film can be easily prepared for the SEM and is not destroyed during the observation process. Both methods, however, only reveal information about a narrow region of the membrane. Therefore, these methods limit us to qualitative evaluation.

For quantitative evaluation of a perturbed PM (i.e., a PM in which the conformation of a single membrane protein, BR, has been structurally modified by various perturbants, such as organic solvents, papain, and light irradiation), Draheim et al. used polarized infrared (IR) spectroscopy to determine the net angle Θ_α between the seven helical segments of the BR polypeptide and the normal to the membrane plane of the PM (i.e., membrane normal). They found that $\Theta_\alpha = 54.735° \pm 0.001°$ for an ethanol-treated PM film and ~0° for a native PM film [5]. This difference in Θ_α is because the secondary structure of the denatured BR molecule is entirely different from that of the native BR molecule due to unfolding [6]. The Θ_α determined using IR agrees well with that obtained using oriented far-ultraviolet (UV) circular dichroism (OCD), ~0° [7]. However, the Θ_α determined from both high-resolution electron cryo-microscopy [1] and electron diffraction (EMD) was ~11° [8]. Gibson and Cassim [7] concluded that the differences in these Θ_α values are due to the drastic differences in the experimental conditions used, especially temperature. The spectral studies were done with fresh hydrated PM films at ambient temperature, whereas the diffraction studies were done with aged glucose-embedded PM at −120 to −268°C, a temperature range in which glucose may not be as benign to biological structures. We therefore decided that an IR method is the most appropriate way to quantify the amount of PM that denatures in an PM LB film.

Here, we present an analysis technique, which we call the spectroscopic surface denaturation quantifying (SSDQ) technique, that uses IR linear dichroism to determine the net native BR ratio κ in a PM film. We then demonstrate this technique by using it to determine the surface area of a single native BR molecule in PM.

2. Materials and methods

2.1. Preparation of PM spreading solution

PM from *H. halobium* was prepared according to the established method in which the spreading solvent was a 33% dimethylformamide aqueous solution [4]. The PM fragments were put into suspension at a BR concentration of 6.0–7.0 mM. The prepared PM spreading solution was never stored for more than a week before use.

2.2. Preparation of PM films and measurement of Π-A isotherms

A conventional LB trough was used for LB film deposition [9]. Pure water used as the subphase was adjusted to pH 3.5 with HCl and was kept at 18.5°C during the experiments. The PM solution was spread onto the subphase by allowing the solution to flow continuously at 50 μl/min through a Teflon tube that touched a point

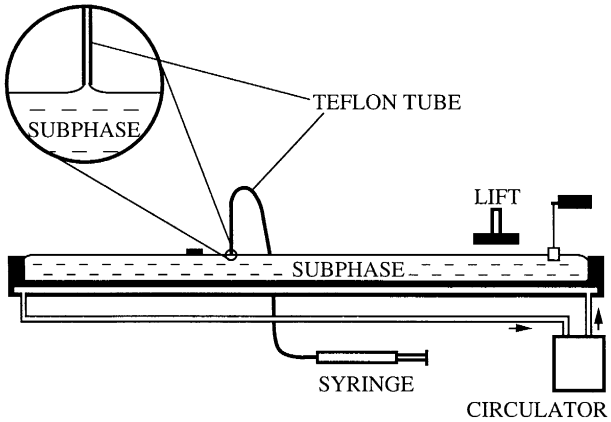

Fig. 1. Side-view of the conventional LB trough used for the LB deposition, showing a Teflon tube touching a point on the surface of the subphase.

on the surface of the subphase (Fig. 1). The volume of spreading solution was changed by the value of the initial coverage, C_i, which was calculated using the equation [4]:

$$C_{\mathrm{i}} = \frac{(\text{Number of BR molecules spread on the subphase}) \times 11.5\,\mathrm{nm}^2}{\text{Area of air/water interface before compression}} \quad (1)$$

where 11.5 nm^2 was the area per BR molecule determined previously from X-ray diffraction analysis [1]. To ensure that the spreading solvent had evaporated sufficiently from the air/water interface, we waited 10 min before starting the compression, and 5 h before starting the preparation of completely denatured PM interface films. During the compression, the shrinkage of the surface area was kept constant at 20 cm^2/min. The compressed interface film was deposited on an indium tin oxide (ITO) glass slide at 25 mN/m by horizontal transfer. After each transfer of a PM interface film, the film on the ITO glass slide was rinsed with pure water and then dried under streaming nitrogen gas.

To prepare a PM film consisting of non-denatured BRs, approx. 1-ml aliquots of the PM spreading solution were layered onto a ITO glass slide and allowed to dry overnight in a sealed chamber at 40% relative humidity.

2.3. Fourier transform infrared (FTIR) spectroscopy

To measure polarized IR reflection-absorption spectra of the PM films on the ITO glass slides, we used an FTIR spectrophotometer (JEOL, JIR-3505) equipped with an MCT absorbance detector (IR-DET101), a polarizer (IR-OPT2), and a reflection unit (IR-RSC11). So that we could analytically determine the indices N_2 of the PM films, in all measurements we used either 60° or 70°as the incident angle of the IR beam to the normal of the sample. The linearly polarized IR beam was either parallel (p-polarized beam, $\|$) or perpendicular (s-polarized beam, $\perp$) to the plane of incidence. One hundred interferograms at 4 cm^{-1} resolution were collected for each film.

To obtain the complex reflective indices $N_3 = n_3\text{-}ik_3$ (where $i = \sqrt{-1}$) of the ITO electrodes, we calculated 'transmission spectra' from the measurement of the reference spectra with an s-polarized beam and from the measurement of the sample spectra with a p-polarized beam on the single-beam mode. A reflection-absorption spectrum of the PM film was obtained from the difference in the spectrum between the PM film/ITO electrode interface and the ITO electrode. Fig. 2 shows a typical reflection-absorption spectra of an PM LB film.

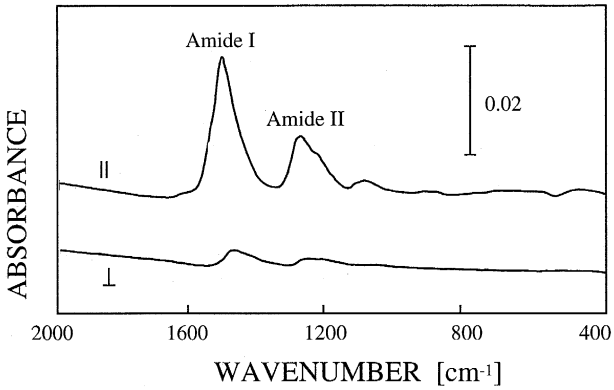

Fig. 2. Typical refraction-absorption spectra of a PM LB film. Upper and lower lines refer to the orientation of the electric vectors parallel (A_p) and perpendicular (A_s) to the plane of incidence, respectively.

2.4. SSDQ technique

IR linear dichroism measurements were taken for PM films whose normal was oriented at an angle of either 60° or 70° ($=\phi_1$) with the IR indicated beam (Fig. 3a). Therefore, the horizontally polarized radiation was polarized in the plane of the film, whereas the vertically polarized radiation was polarized either 60° or 70° out of the plane of the film. The BR net helical tilt angle (the angle between the polypeptide segments and the membrane normal) in PM films, Θ_α, was determined by the following equation, which is based on an equation derived by Draheim et al. [5] in a manner analogous to the derivation by Rothschild and Clark [10]:

$$R = \frac{A_p}{A_s} = \frac{|E_p|^2}{|E_s|^2}\left\{1 + 3\cos^2\xi \frac{f_I S_I + f_{II} S_{II}}{f_I(1 - S_I) + f_{II}(1 - S_{II}) + + f_u}\right\} \tag{2}$$

where R is the observed dichroic ratio at a given wavenumber, A_p is the absorbance of the vertically polarized radiation E_p at the PM LB film/ITO interface, A_s is the absorbance of the horizontally polarized radiation E_s, ξ is the angle between the vertically polarized radiation vector and the film normal (Fig. 3 b), f_I, f_{II} and f_u are the fractions of residues in the α_I-helix, α_{II}-helix and aperiodic secondary structure, respectively, and S_I and S_{II} are total order parameters for the α_I-helix and α_{II}-helix, respectively. (This equation is described in detail in Appendix A.)

The ratio κ is defined as the number of native BR molecules to the total number of BR molecules in a PM film, and was determined by the following equation (see Appendix B for details):

$$\kappa = \frac{P_2(\cos\Theta_\alpha) - P_2(\cos\Theta_{\alpha D})}{P_2(\cos\Theta_{\alpha N}) - P_2(\cos\Theta_{\alpha D})}. \tag{3}$$

where $\Theta_{\alpha N}$ is the net angle between polypeptide segments and the membrane normal in native BR in the PM film, $\Theta_{\alpha D}$ is that in denatured BR (Fig. 3c), and P_2 is the Legendre polynomial of the second order. Note that for convenience, we have summarized these various angles and their notations in Table 1.

Because there may not be perfect orientation of the PM fragments in the PM films, we must consider the mosaic spread tilt angle, Θ_m, which is the angle between the PM film normal and the PM fragment normal. In previous calculations, Θ_m values have been assumed to be in the range of 1–15° [5,8,10]. However, in our data analysis, we need not assume a Θ_m value. The κ was determined by the following equation, in which we used

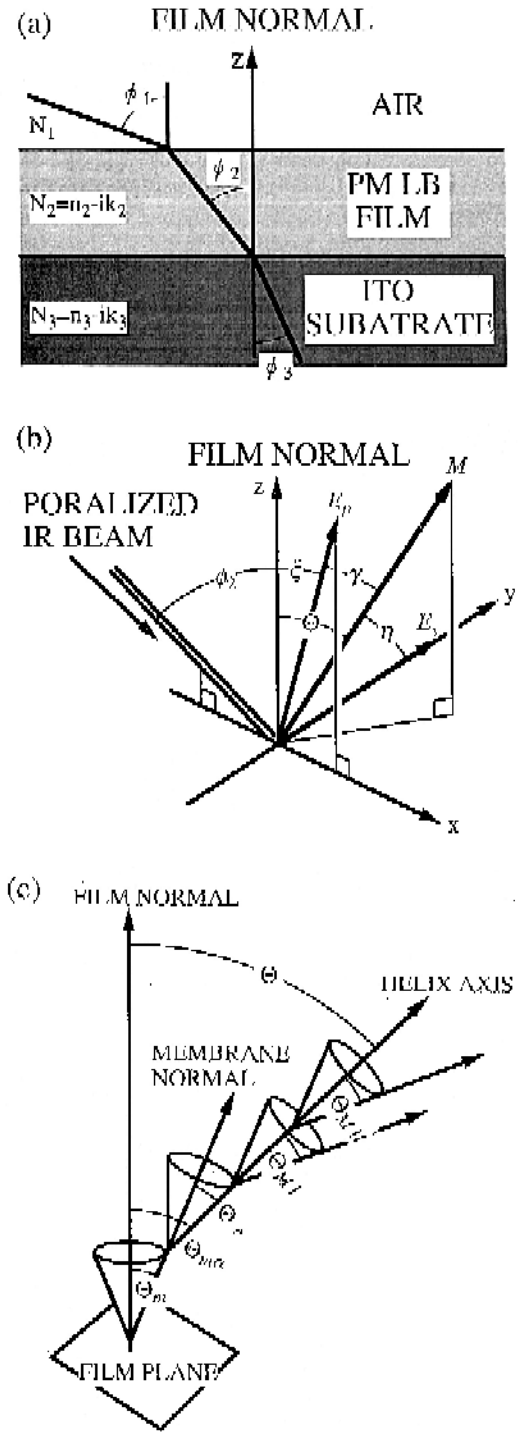

Fig. 3. (a) Reflection-absorbance at a three-phase (i.e., air, PM film, and ITO substrate) plane-bounded system. Here, N_1, N_2 ($= n_2$-i k_2), and N_3 ($= n_3$-i k_3) are the reflective indices of air, a PM LB film, and ITO, respectively. (b) Geometry for the electric vectors, E_p and E_s, of the incident polarized IR beam, and the averaged vector of transition moment, M, originating in amide groups of polypeptides of BR molecules at the interface of a PM film and ITO substrate. Here, η, γ, ξ, Θ, and ϕ are the angles. (c) Geometry of the four nested axially symmetric cones utilized for data analysis of the polarized IR spectra of an oriented PM film.

Table 1

Symbol of Angle	Definition
Θ_{α}	BR net helical segmental tilt angle between the polypeptide segments and the normal of the PM fragment in a PM LB film.
$\Theta_{\alpha N}$	Native BR net helical segmental tilt angle between the polypeptide segments and the normal of the PM fragment.
$\Theta_{\alpha D}$	Unfolded BR net helical segmental tilt angle between the polypeptide segments and the normal of the denatured PM in a PM LB film. [a]
Θ_{m}	Angle between the normal of a PM and that of a PMLB film (or the normal of the ITO glass slide).
$\Theta_{m\alpha}$	BR net helical segmental tilt angle between the polypeptide segments and the normal of a PM LB film (or the normal of the ITO glass slide).
$\theta_{m\alpha N}$	Native BR net helical segmental tilt angle between the polypeptide segments and the normal of PM LB film (or the normal of the ITO glass slide).
$\Theta_{m\alpha D}$	Unfolded BR net helical segmental tilt angle between the polypeptide segments and the normal of the denaturated PM in a PM LB film (or the normal of the ITO glass slide).
Θ_{MI}, (Θ_{MII})	Angle between the amide transition dipole moment, M, and the α_I, (α_{II})-helix axis.

[a] An unfolded BR resulted from the surface denaturation at the air/water interface maintained the α-helical conformation.

the angles $\Theta_{m\alpha}$, $\Theta_{m\alpha N}$, and $\Theta_{m\alpha D}$ between polypeptide segments and the film normal (see Appendix B for details):

$$\kappa = \frac{P_2(\cos\Theta_{m\alpha}) - P_2(\cos\Theta_{m\alpha D})}{P_2(\cos\Theta_{m\alpha N}) - P_2(\cos\Theta_{m\alpha D})}. \tag{4}$$

Note that κ determined by Eq. (4) agrees well with that determined by Eq. (3) (see appendix B for a detailed explanation).

3. Results and discussion

3.1. Π-A isotherms

Fig. 4 shows that the Π-A curves shifted to the small molecular area side as C_i increased. Furuno et al. found (using the time dependence of the shape and packing of the PM fragments at $C_i = 0.25$) that PM fragments

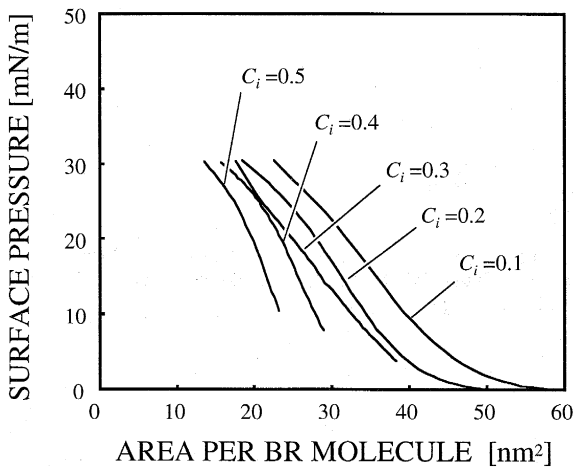

Fig. 4. Dependence of Π-A isotherms for BR molecules on initial coverage C_i.

rapidly melt at an air/water interface when the C_i is low and that unfolded BR molecules exist in the intermembrane region [6]. Their results indicate that the molecular area of unfolded BR at an air/water interface is larger than that of native BR.

3.2. BR net helical segmental tilt angle Θ_α

We determined $\Theta_{m\alpha}$ between the polypeptide segments and the film normal for PM LB films that were prepared under several values of C_i. Fig. 5 shows that the $\Theta_{m\alpha}$ decreased as C_i increased. The overall net angle was $\Theta_{m\alpha}$ and not Θ_α. In previous calculations, Θ_m was assumed to be in the 1–15° range [5,10]; for example, Draheim et al. assumed an Θ_m value of 10°, because their determination of Θ_α requires an assumed value for Θ_m. Here, as described in Appendix B, in our determination of κ there is no need for such an assumed value.

We determined a value of 19.8° for $\Theta_{m\alpha N}$, and a value of 55.0° for $\Theta_{m\alpha D}$. When we assumed that Θ_m was 10°, $\Theta_{\alpha N}$ was 17° and $\Theta_{\alpha D} = 55°$. The $\Theta_{\alpha N}$ value of 17° determined here differs from the value of $\sim 0°$ determined in other studies [5]. Here, the PM fragments were suspended in a 33% dimethylformamide aqueous solution, which had the advantage that PM fragments or BR molecules were sufficiently stable [4]. Draheim et al. reported that conformational changes occur in the BR secondary structure, especially the helical tilt angle, which they verified by various experimental perturbations, such as adding solvents during the PM preparation process and varying the temperature of the film during the observation [5]. We suspect that the perturbations caused by the spreading solvent (i.e., dimethylformamide) and the surface tension at the air/water interface gave rise to the difference between 17° and 0° for $\Theta_{\alpha N}$. Furthermore, the value of $54.735° \pm 0.001°$ for $\Theta_{\alpha D}$ was determined for an ethanol-treated PM film, which is considered an ideal random state standard [5]. As mentioned earlier, results from OCD spectroscopy of ethanol-treated PM films suggest that even after the ethanol treatment the α-helical structure is maintained but randomized with respect to the membrane normal [11]. The value of 55° that we obtained here for completely denatured PM LB films agrees well with the $54.735° \pm 0\ 001°$ for ethanol-treated films. Furuno et al. concluded that BR molecules (of the PM) unfold on an air/water surface [6]; however, no detailed information was obtained on the conformational changes in the BR secondary structure.

Both the 222-nm and $\sim$207-nm bands are usually evident in CD spectra originating from α-helical conformation consisting of a typical protein (i.e., myoglobin molecules), whereas only the 222-nm band is evident in that originating from two types of α-helix, (α_I-helix and the α_{II}-helix) of a native BR molecule [11]. Therefore the difference in the CD spectra originates from α_{II}-type helical conformation in the BR molecules. We measured the OCD spectra of PM cast films and completely denatured PM LB films while the incident light

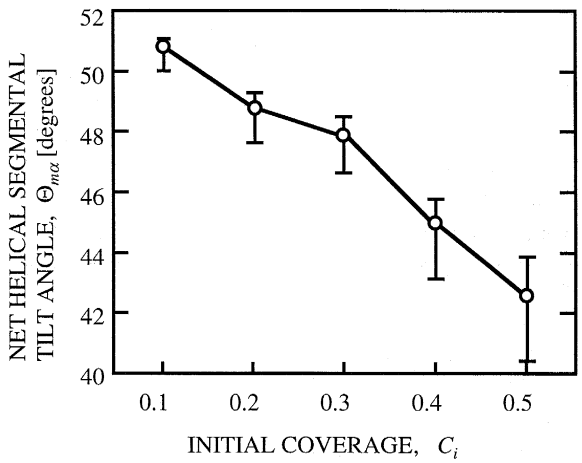

Fig. 5. Dependence of the net helical segmental tilt angle $\Theta_{m\alpha}$ between the seven helical segments of the BR polypeptide and the normal to the film plane of PM LB films on initial coverage C_i.

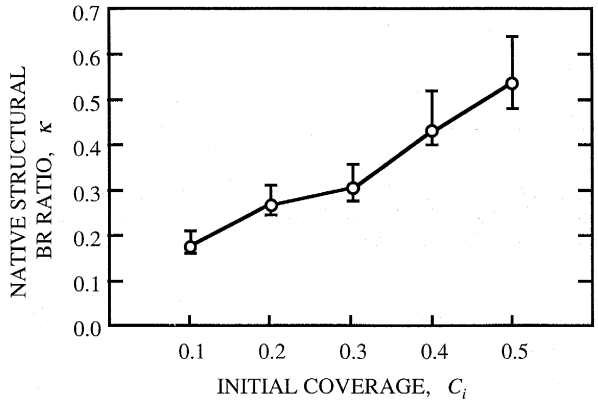

Fig. 6. Dependence of the ratio κ (the number of BR molecules that maintained the native conformation after preparation of a PM film to the total number of BR molecules in the PM LB film) on initial coverage C_i.

was parallel to the film normal (data not shown). The OCD spectrum of the PM cast film showed only the ~222-nm band, whereas that of the completely denatured PM LB films showed both the ~220-nm and ~207-nm bands. This suggests that the α-helical conformation of unfolded BR in the completely denatured PM LB films was maintained during the surface denaturation at the air/water interface, while the α_{II}-type-like helical conformation was changed to the α_I-type-like conformation. Therefore, a value of 55° for $\Theta_{\alpha D}$ for the completely denatured PM LB films suggests that the net tilt angle of the α-helical segment of the BR was completely randomized, because in viewpoint of the helix geometry the net tilt angle of oriented segments completely random to the incident light should be theoretically 54.736°. We conclude that the perturbed BR due to surface denaturation produced the same conformational change as does ethanol treatment. That is, due to the surface denaturation, the helical segments of BR are completely randomized in a PM interface film.

3.3. Non-denatured BR ratio κ in PM films

Fig. 6 shows the C_i dependency on the non-denatured BR molecules ratio κ in the PM LB films. The increase in κ with increasing C_i shows that the high concentration of the PM fragments on the air/water surface suppressed the surface denaturation of the PM.

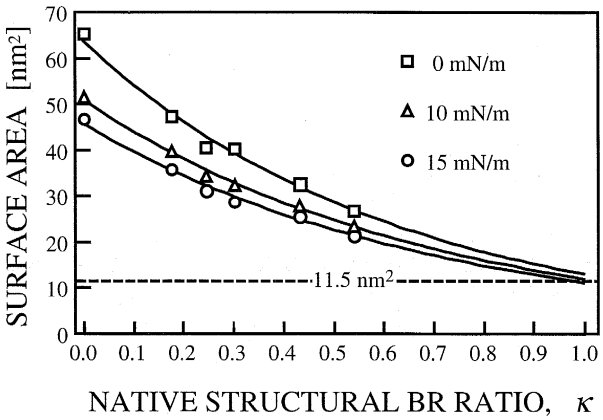

Fig. 7. Dependence of the non-denatured BR ratio κ on the surface area per BR molecule on an air/water interface under a constant surface pressure of 0 (□), 10 (△), or 15 (○) mN/m. Data for each constant surface pressure were fitted with an exponential curve. The extrapolated area at $\kappa = 1$ was ~11.5 nm^2.

Fig. 7 shows the dependency of κ on the surface area occupied by a single BR molecule on the air/water interface under various levels of constant surface pressure. By fitting the data with an exponential function and then extrapolating to $\kappa = 1.0$, we determined that the surface area of a single non-denatured BR molecule was ~ 11.5 nm^2 in the films prepared under a surface pressure range of 11–15 mN/m, which is less than the pressure that causes a PM interface film to collapse. This extrapolated value for the surface area agrees well with the value of 11.5 nm^2 determined by EMD [1].

It is found that the present SSDQ technique is a useful method for determining the κ for PM LB fiims. We think that the SSDQ technique is also applicable to other types of protein consisting of α-helical conformation.

4. Conclusions

In this paper, we developed the SSDQ technique, which uses linear IR dichroism, to quantitatively evaluate the ratio κ of non-denatured BR molecules in PM LB films. Using this technique, we found that the conformational change after surface denaturation of BR was the same as that caused by an ethanol treatment. By extrapolation of κ vs. C_i data, we found that the surface area of a single non-denatured BR molecule was ~ 11.5 nm^2, which is consistent with that determined by EMD. These results demonstrate conclusively that this technique is effective in quantifying the value of κ for PM LB films. Finally, the present SSDQ technique is also applicable to other types of protein consisting of α-helical conformation.

Appendix A

Absorbance A_p and A_s as measured for the geometry shown in Fig. 3b are

$$A_p = \left\langle \left(E_p \cdot M \right)^2 \right\rangle_f = |E_p|^2 \langle \cos^2 \gamma \rangle_f \tag{A.1.p}$$

$$A_s = \left\langle \left(E_s \cdot M \right)^2 \right\rangle_f = |E_s|^2 \langle \cos^2 \gamma \rangle_f \tag{A.1.s}$$

where M is a vectorial transition moment and $\langle \rangle_f$ is an average over the distribution $f(\Theta,\Phi)$ of orientation M. We assumed that the sample was isotropic with respect to orientation in the plane (i.e., f was independent of Φ [5]).

The relation between the dichroic ratio R and the angle Θ of the total transition moment M is

$$R = \frac{A_p}{A_s} = \frac{|E_p|^2}{|E_s|^2} \left\{ 1 + 3\cos^2 \xi \frac{\langle P_2(\cos \Theta) \rangle_f}{1 - \langle P_2(\cos \Theta) \rangle_f} \right\} \tag{A.2}$$

where $P_2(x)$ is the second Legendre function, namely, an order parameter.

We calculate the transition coefficients t_{p12} and t_{s12} at the air/film interface, and the reflection coefficients r_{p23} and r_{s23} at the film/ITO-substrate interface as

$$t_{p12} = \frac{2\epsilon_2 \xi_1}{\epsilon_2 \xi_1 + \epsilon_1 \xi_2},\ t_{s12} = \frac{2\xi_1}{\xi_1 + \xi_2}, \tag{A.3}$$

$$r_{p23} = \frac{\epsilon_3 \xi_2 - \epsilon_2 \xi_3}{\epsilon_3 \xi_2 + \epsilon_2 \xi_3},\ r_{s23} = \frac{\xi_2 - \xi_3}{\xi_2 + \xi_3}, \tag{A.4}$$

where $\epsilon_m = N_m^2$ and $\xi_m = N_m \cos\phi_m$ (m = 1,2,3) as shown in Fig. 3a. We can then express the factors $|E_p|^2/|E_s|^2$ and $\cos^2 \xi$ in Eq. (A.2) as

$$\frac{|E_p|^2}{|E_s|^2} = \frac{|t_{p12}|^2\left\{1 + |r_{p23}|^2 - 2\left(1 - 2\sin^2\alpha/|N_2|^2\right) Re(r_{p23})\right\}}{|t_{s12}|^2\left\{1 + |r_{p23}|^2 + 2\,Re(r_{p23})\right\}}, \tag{A.5}$$

$$\cos^2\xi = \frac{\left\{1 + |r_{p23}|^2 + 2\,Re(r_{p23})\right\}\sin^2\alpha/|N_2|^2}{1 + |r_{p23}|^2 - 2\left(1 - 2\sin^2\alpha/|N_2|^2\right) Re(r_{p23})}. \tag{A.6}$$

The α-helices for BR have equal α_I- and α_{II}-structural characteristics. By taking this into account, Draheim et al. analyzed the BR net helical segmental tilt angles, Θ_α [5]. Again, we assumed here that the sample was isotropic with respect to orientation in the plane of the substrate. To obtain $\langle P_2(\cos\Theta)\rangle_f$, we assumed the distribution $f(\Theta)$ of the orientation M to be a δ function, and f_I, f_{II} and f_u to be the fraction of residues in α_I-helix, α_{II}-helix, and aperiodic secondary structure, respectively. Therefore,

$$\langle P_2(\cos\Theta)\rangle_f = f_I S_I + f_{II} S_{II}, \tag{A.7}$$

where S_I and S_{II} are the total order parameters of the α_I-helix and α_{II}-helix, respectively. In our calculations, f_I, f_{II} and f_u were assumed to be 0.4, 0.4, and 0.2, respectively [5].

S_I and S_{II} were determined by

$$S_I = S_m S_\alpha S_{MI}, \tag{A.8.1}$$

$$S_{II} = S_m S_\alpha S_{MII}, \tag{A.8.2}$$

where S_m, S_α, S_{MI}, and S_{MII} are the mosaic spread, helix axis, α_I-amide transition dipole moment, and α_{II}-amide transition dipole moment order parameters, respectively, which describe four nested axially symmetric cones as illustrated in Fig. 3c. The angles of these cones are calculated using

$$S = \frac{3\cos^2\Theta - 1}{2} \tag{A.9}$$

where S is S_m, S_α, S_{MI} or S_{MII} and Θ is Θ_m, Θ_α, Θ_{MI} or Θ_{MII}. Here, Θ_m is the mosaic spread tilt angle (the angle between the film normal and the membrane normal); Θ_α is the α-helical axis tilt angle (the angle between the helical axis and the membrane normal); and Θ_{MI} and Θ_{MII} are the amide transition dipole moment angles for the α_I- and α_{II}-helices, respectively (the angle between the transition moment and the helix axis). Draheim et al. estimated Θ_{MI} to be 22°–29° for amide I and 82°–88° for amide II, and Θ_{MII} to be 42°–53° for amide I and 86°–91° for amide II [5]. In our calculation, we used the median of either Θ_{MI} (i.e., 25.5° for amide I or 85° for amide II) or Θ_{MII} (i.e., 47.5° for amide I or 88.5° for amide II).

Appendix B

According to SEM observations of PM interface films transferred onto supports, the film looked like a ‘mosaic’ of PM fragments [3]. SEM observations of pure PM interface films showed that the PM denatures, in other words, BR unfolds on the air/water interface, and the unfolded BR molecules and the membrane lipids spread over the intermembrane space [4]. In our calculation, we assumed constant f_I, f_{II} and f_u, which are the fractions of residues in the α_I-helix, α_{II}-helix, and aperiodic secondary structure, respectively.

Again, we defined κ as the ratio of the number of BR molecules with native conformation to that of BR molecules on the air/water surface. The absorption of the p-polarized and s-polarized IR irradiations (A_p and A_s, respectively) is the sum of the absorption originating from the transition moments for the polypeptide amide

groups, that is amide I and amide II of the non-denatured BR molecules, and the absorption of the unfolded BR molecules.

We defined A_p and A_s of the native PM films consisting of only native conformational BR molecules as A_{pN} and A_{sN}, and those of the surface denatured PM LB films consisting of only unfolded BR molecules as A_{pD} and A_{sD}. We can then express the relationship among these absorptions as

$$A_p = \kappa A_{pN} + (1-\kappa) A_{pD}, \tag{B.1.1}$$

$$A_s = \kappa A_{sN} + (1-\kappa) A_{sD}. \tag{B.1.2}$$

We applied a derivation that is analogous to that described in appendix A to these two equations, and derived the following equation after comparing the resultant form with Eq. (A.2):

$$\langle P_2(\cos\Theta)\rangle_f = \kappa(f_I S_{IN} + f_{II} S_{IIN}) + (1-\kappa)(f_I S_{ID} + f_{II} S_{IID}). \tag{B.2}$$

S_{IN}, S_{IIN}, S_{ID} and S_{IID} were determined as

$$S_{IN} = S_m S_{\alpha N} S_{MI}, \tag{B.3.1}$$

$$S_{IIN} = S_m S_{\alpha N} S_{MII}, \tag{B.3.2}$$

$$S_{ID} = S_m S_{\alpha D} S_{MI}, \tag{B.3.3}$$

$$S_{IID} = S_m S_{\alpha D} S_{MII}, \tag{B.3.4}$$

where S_{aN} and S_{aD} are the native BR helix axis and the unfolded BR helix axis order parameters, respectively. Consequently, from Eq. (A.7) and Eq. (B.2), we have the following equation for κ:

$$\kappa = \frac{S_\alpha - S_{\alpha D}}{S_{\alpha N} - S_{\alpha D}}. \tag{B.4}$$

κ does not change even when the order parameters in Eq. (B.4), S_α, S_{aN} and S_{aD}, are replaced by the order parameters with mosaic spread, $S_m S_\alpha$, $S_m S_{aN}$ and $S_m S_{aD}$, respectively. Therefore, to determine κ, we can use the BR net helical tilt angles, $\Theta_{m\alpha}$, $\Theta_{m\alpha N}$ and $\Theta_{m\alpha D}$ (between the polypeptide segments and the film normal to the ordinary PM LB film, the native PM film, and the denatured PM films, respectively). The relationship between these order parameters and these angles are as follows:

$$S_m S_\alpha = P_2(\cos\Theta_{m\alpha}), \tag{B.5.1}$$

$$S_m S_{\alpha N} = P_2(\cos\Theta_{m\alpha N}), \tag{B.5.2}$$

$$S_m S_{\alpha D} = P_2(\cos\Theta_{m\alpha D}). \tag{B.5.3}$$

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