Characterization of Langmuir-Blodgett Films of Rhodopsin: Thermal Stability Studies

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ABSTRACT Two-dimensional close packing of purified bovine rhodopsin, made by the Langmuir-Blodgett technique, was characterized by small angle x-ray scattering and nanogravimetric measurements. The area occupied by a molecule of rhodopsin in the film was ~1100 Å^2 and the periodicity of the layers resulted in 59 Å. The circular dichroism measurements showed that bleached rhodopsin in Langmuir-Blodgett film had high thermal stability, in fact, reaching a temperature of 150°C without a loss of the secondary structure. Moreover, when the film was made up in the dark, rhodopsin maintained its stability up to at least 200°C and its characteristic absorbance peak at 500 nm up to about 90°C.

INTRODUCTION

Thin films of light-sensitive proteins performed by the Langmuir-Blodgett (L-B) method are an expanding field with exciting applications such as optical information processing and molecular electronic devices. The close packing of protein molecules in two-dimensional, highly ordered, crystal-like structure could dramatically change some of the chemicophysical properties commonly known for protein in solution. For instance, high thermal stability has recently been reported for films of photosynthetic reaction centers (Nicolini et al., 1993), anti-insulin antibodies (Erokhin et al., 1994), and bacteriorhodopsin (Shen et al., 1993). The reaction center in L-B films preserved its secondary structure up to 160°C and its function up to 80°C whereas bacteriorhodopsin in dry films had a melting transition and denaturation at ~140°C.

We also considered that vertebrate rhodopsin could be utilized in this field, as it is a well known intrinsic membrane protein structurally similar to bacteriorhodopsin and, moreover, some properties of rhodopsin films are already known (Montal et al., 1977; Korenbrot and Pramik, 1977; Salesse et al., 1990). Vertebrate rhodopsin spans the disk membranes of the retinal rod outer segment with seven α-helices. Its chromophore, 11-*cis* retinal is located in a hydrophobic pocket between the helices and covalently attached to lysine 296 via a Schiff-base linkage. Rhodopsin has an absorbance maximum at ~500 nm. Upon absorption of light, 11-*cis* retinal is first isomerized to all-*trans* retinal and then detached from the apoprotein opsin by the hydrolysis of the Schiff-base, causing a decrease in the absorbance maximum (bleaching). In vivo, the absorption of a photon by a rhodopsin molecule triggers the cyclic GMP cascade of phototransduction, which constitutes the molecular events of vertebrate vision.

The advantage of making thin films with rhodopsin rather than with bacteriorhodopsin is that rhodopsin can be extracted by detergent from the membrane without loosing most of its properties, whereas the molecules of bacteriorhodopsin seem unstable in detergent (Hampp, 1993). For this reason, films of bacteriorhodopsin are usually made by using natural purple membranes. By utilizing purified rhodopsin instead, solubilized with an appropriate detergent that can be eliminated during the assembly of the L-B film, mono- or multimolecular layers can be obtained made up mainly of rhodopsin. In these layers rhodopsin molecules could be assembled closer together than they are in native membranes, so as to build up nontraditional photosensitive material, possibly capable of carrying processes of light-dependent transfer of electrons and protons with high quantum efficiency.

Another advantage is that rhodopsin in dry films, at room temperature, has photoconvertible stable states (Wald et al., 1950; Korenbrot and Pramik, 1977), whereas bacteriorhodopsin has some limitations arising from different photochemical side reactions (Oesterhelt et al., 1991).

In this paper we report a successful attempt to build two-dimensional films made up of mainly bovine rhodopsin by the L-B method. In these films rhodopsin seems organized as in native membranes with the cylindrical axe perpendicular to the plane of the film but with the molecules assembled very close together, as indicated by the area occupied per molecule, which is close to that calculated for rhodopsin without the surrounding lipids. Moreover, the rhodopsin in these films shows a high thermal stability, reaching in fact a temperature of 150°C in the light and 200°C in the dark, with a negligible loss in its secondary structure and preserving its characteristic absorbance peak at 500 nm up to ~90°C.

MATERIALS AND METHODS

Preparation of rhodopsin solutions

Rhodopsin was purified from rod outer segments (ROS) of bovine retinas. In a typical preparation, 20 bovine retinas were dissected from eyes

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explanted no more than 20 min after the death of the animals at a local slaughter house. The ROS were isolated by shaking the retinas for 30 s in 35% (w/v) sucrose in 1 ml of Ringer solution (157 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 6.7, at 4°C) per retina. The material was then centrifuged at 6000 rpm for 10 min. This procedure was repeated twice and the two supernatants were put together and diluted with 2 vol of Ringer solution. After centrifugation at 4000 rpm for 10 min, the pellet containing ROS was collected. Disk membranes containing rhodopsin were isolated from the ROS following the method of Smith et al. (1975) but partially modified. The ROS were allowed to burst in an aqueous hypotonic solution of 5% Ficoll (Sigma Chemical Co., St. Louis, MO) for 3 h at 4°C. Osmotically intact disks were then separated from the plasma membrane and other debris by centrifuging them in an SW-41 Beckman rotor at 25,000 rpm for 2 h. After centrifugation, the disk suspension floating at the Ficoll-air interface was collected, washed twice in 10 vol of distilled water, and collected by centrifugation at 18,000 rpm for 30 min. Rhodopsin was brought into solution by shaking the isolated disks in 1.5% β-octylglucoside (Sigma Chemical Co.). The suspension was centrifuged at 13,000 rpm for 30 min and the pellet was discarded. Purification of rhodopsin and removal of phospholipids was carried out by affinity chromatography on a concanavalin A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column (16 × 300 mm), following the method of Litman (1982). Spectral measurements of the purified rhodopsin were performed with a Jasco 7800 UV/VIS spectrophotometer and gave a A280/A500 ratio of 1.8. The rhodopsin concentration was determined by measuring the change in absorbance at 500 nm after illumination in the presence of 0.1 M hydroxylamine, with an extinction coefficient of 40,600 M^{-1} cm^{-1} at 500 nm and a molecular weight of 39,000 (Dratz and Hargrave, 1983).

Formation and deposition of rhodopsin L-B films

L-B films were prepared and studied in a L-B trough (MM-MDT Co., Moscow, Russia) of 240- × 100-mm size and 300-ml volume. The instrument has two moving barriers between which films are compressed and expanded. The surface pressure measurements were made by a Wilhelmy balance with a sensitivity of 0.05 mN/m. The trough was filled with a subphase of 0.5 M NaCl.

The following procedures were carried out in room light, unless otherwise specified. A small amount (20 μl) of rhodopsin (1.5 mg/ml) in 0.75% β-octylglucoside was spread at the air-water interface causing the formation of a monolayer that was compressed by the moving barriers to reach the surface pressure of the deposition (Lvov et al., 1991; Hann, 1990). The film was then tranferred onto solid substrates with the Langmuir-Schaefer technique (1938) at 35 mN/m surface pressure. Samples for circular dichroism (CD) and spectrophotometric measurements were deposited onto standard quartz slides. Samples for small-angle x-ray measurements were prepared by deposition onto silicon substrates. Quartz slides and silicon substrates were cleaned in warm sulfuric acid solution saturated with potassium dichromate, washed in distilled water, and then dried under nitrogen flux.

Nanogravimetric measurements

The surface density of rhodopsin on the L-B films was measured by successive layer depositions on a quartz piezoelectric resonator (resonance frequency of 10 MHz) of a homemade nanogravimetric gauge (Facci et al., 1993). The shift in resonance frequency Δf after deposition is correlated to the surface density Δs of the mass deposited, by the Sauerbrey equation: $\Delta s = -K\Delta f$, where K is a constant, whose value was estimated to be ~0.0295 ng Hz^{-1} mm^{-2} according to the procedure reported in the literature (Facci et al., 1993).

X-ray diffractometric measurements

X-ray diffractometric measurements of rhodopsin L-B films (30 layers) were carried out by means of a small-angle x-ray diffractometer (AMUR-K made by Institute of Crystallography, Russian Academy of Sciences, Moscow, Russia) equipped with a linear position-sensitive detector (Mogilevski et al., 1984). Cu Kα (1.54 Å) radiation was used. The angular resolution of the detector was 0.01°. The sample was rotated with respect to the x-ray beam and the scattered radiation was collected by the detector in the angular range of 0°-7°. Measurements were performed at room temperature.

Circular dichroism measurements

A Jasco J710 spectropolarimeter was used in the range of wavelengths 185–260 nm. Measurements with rhodopsin solution (0.01 mg/ml) were performed in the range of temperature between 25 and 110°C, with a Jasco PTC-343 Peltier type thermostatic cell holder located inside the spectropolarimeter. The sample was maintained at the indicated temperature for 10 min. The CD spectrum of a control sample of 0.75% β-octylglucoside was substracted from the CD spectrum of rhodopsin solution. The detergent did not show any optical activity.

Measurements with rhodopsin L-B films were performed in the range between 25 and 200°C. In this case, for temperatures >110°C, the sample was preheated in the oven at the necessary temperature for 10 min and then analyzed at the fixed temperature of 110°C. A control sample was made by spreading a solution of 2% β-octylglucoside on a quartz slide and dried, as a film of this detergent was impossible to deposit because of its low collapse pressure (~10 mN/m). The control showed no optical activity and its CD spectrum was indistinguishable from that of the quartz slide, which is usually substracted from the CD spectra of samples.

RESULTS

L-B films of rhodopsin were prepared as described in Materials and Methods by spreading rhodopsin, solubilized into 0.75% β-octylglucoside, at the air-water interface. As this preparation was performed in room light, the term opsin will be used instead of rhodopsin. The resulting monolayer was compressed by two moving barriers and consequently the surface pressure of the film increased from 20 to 35 mN/m. Depositions were then performed at a surface pressure of 35 mN/m. The surface density of the monolayer was measured by depositing opsin L-B film (up to 10 layers) on the quartz resonator of a nanogravimetric gauge. From the data shown in Fig. 1, the result of the surface density of the monolayer was ~5.9 ± 1.3 ng/mm^2 (SD of 10 measurements). Assuming that most of the material deposited was opsin (see Discussion), the area per molecule was deduced from this value. The molecular mass of bovine rhodopsin

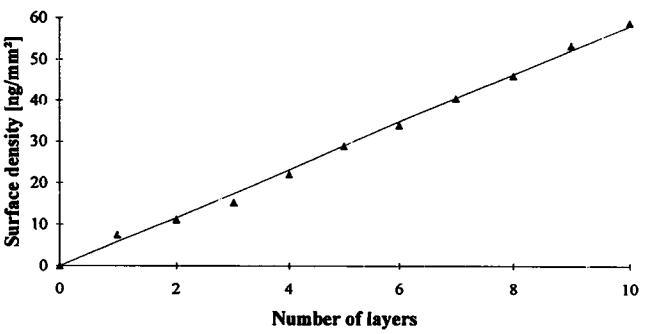

FIGURE 1 Nanogravimetric measurements of deposition of opsin L-B layers.

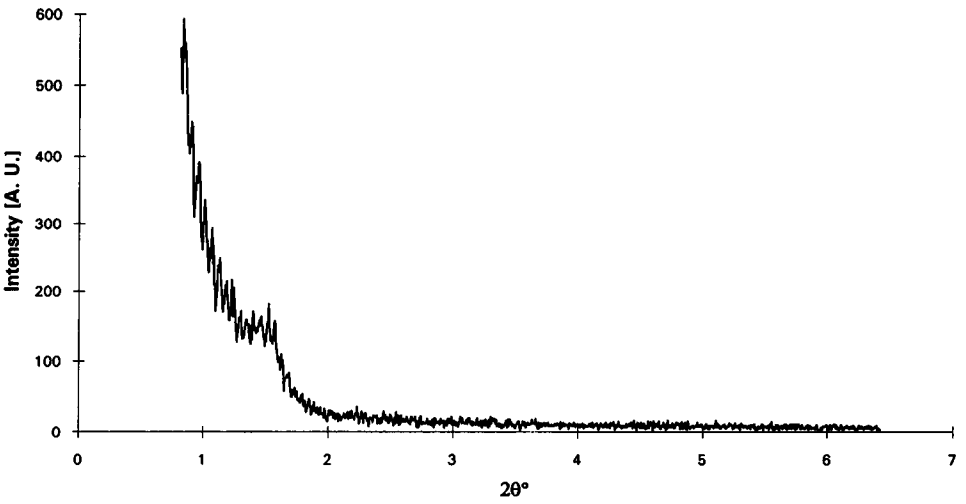

FIGURE 2 Low-angle x-ray diffractometric measurements of 30 layers of opsin L-B film.

being 39,000 g/mol (Dratz and Hargrave, 1983), which is $\sim 6.5 \times 10^{-20}$ g/molecule, the number of opsin molecule per square millimeter resulted in

$$\frac{5.9 \times 10^{-9} \text{g/mm}^2}{6.5 \times 10^{-20} \text{g/molecule}} = 0.908 \times 10^{11} \text{ molecules/mm}^2$$

and therefore the area occupied by one molecule of opsin in the L-B film was 1.1×10^{-11} mm^2 = 1100 Å^2. This value is close to that of the area occupied by a molecule with a diameter of 40 Å, such as the molecule of rhodopsin, which is thought to have a cylinder shape of roughly 40 Å × 60 Å, with the larger dimension perpendicular to the plane of the disk membrane (Dratz and Hargrave, 1983; Schertler et al., 1993).

Fig. 2 shows the results of low-angle x-ray scattering measurements. The x-ray diffraction pattern of 30 layers of opsin L-B film at room temperature shows a wide reflection at $\sim 2\theta = 1.5$ from which, using the Bragg relation, a periodicity of ~59 Å ± 3 Å was obtained. This value is in agreement with the longer dimension of rhodopsin, ~60–65 Å, as reported in the literature (Dratz and Hargrave, 1983), which suggests that the assembly of opsin in L-B films could be similar to that in disk membranes.

Fig. 3 shows CD spectra of opsin in L-B films and in solution. The comparison between the two spectra as well as the data shown in Tables 1 and 2 at 25°C suggest that the L-B film organization caused only small modifications of the secondary structure of opsin.

The thermal stability of the secondary structure of opsin in the L-B film was studied by CD in the range between 25 and 200°C, as shown in Fig. 4. CD spectra were used for computation of the percentage of α-helix, β-sheet, β-turn, and random coil of rhodopsin by a modified Hennessey-Johnson procedure (Carrara et al., 1992). Although these calculations are based on a set of standard globular proteins (Chang et al., 1978), they merely indicate (see Table 1) that opsin in the L-B film mantained its secondary structure up to at least 150°C with no significant loss of the the α-helix structure, as a partial unfolding process was evident only at 175°C. The small changes in the secondary structure shown from 25 to 150°C were irreversible, as there was no change

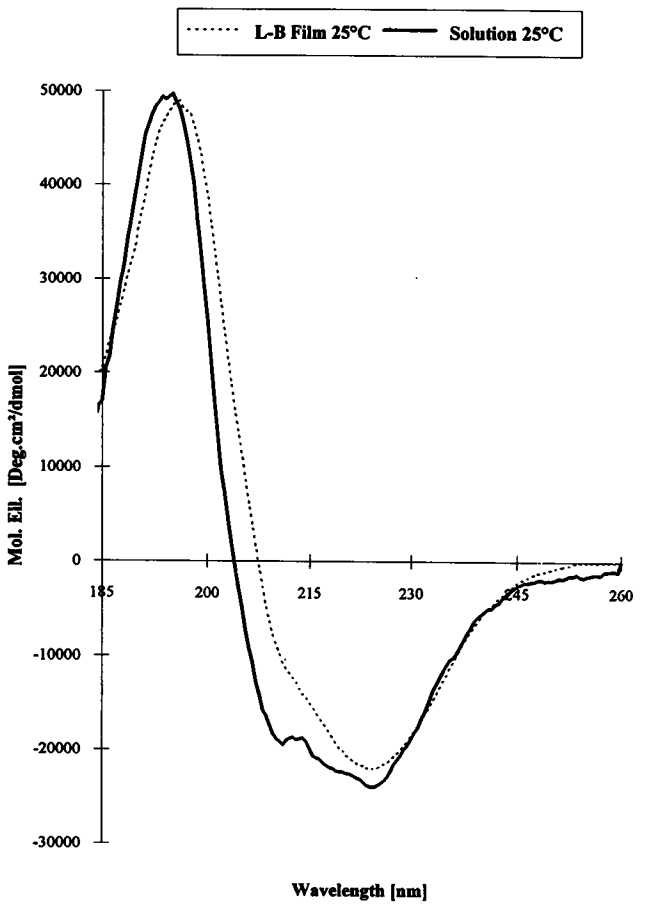

FIGURE 3 Comparison between CD spectra of opsin in solution (0.01 mg/ml) and in L-B film (30 layers) at 25°C. The calculation of molar ellipticity in the film was performed by normalizing the absorbance of the film to that of the solution according to the approach suggested by Nicolini et al. (1993).

TABLE 1 Temperature dependence of rhodopsin secondary structure in L-B film

Temperature (°C)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
25	68	13	7	12
100	68	14	4	14
125	66	10	5	19
150	66	7	5	22
175	58	5	1	36
200	50	3	5	42

Percentage of α-helix, β-sheet, β-turn, and random coil calculated from CD spectra of rhodopsin L-B film (30 layers) at different temperatures.

in the CD spectra of the heated samples when they were brought to room temperature.

When the preparation and deposition of rhodopsin films were made in dim red light, the secondary structure of the protein remained unchanged up to at least 200°C (see Fig. 5).

As a comparison, the thermal stability of the secondary structure of bleached rhodopsin in solution is shown in the CD spectra of Fig. 6 and the calculations of the percentage of secondary structure, based on these spectra, are shown in Table 2. The results indicate that opsin in solution as in L-B film had an α-helix content of 68% at 25°C, close to the value of ~62% reported in the literature (Shichi, 1971), which decreased gradually from 45 to 95°C. At 95°C the α-helix content was 40% and the random coil 47%, whereas opsin in L-B film at 100°C showed negligible variation of its secondary structure.

The order of opsin molecules in the direction normal to the film plane was also maintained in the heated multilayers, as suggested by the low-angle x-ray scattering measurements shown in Fig. 7. The Bragg reflection at $\sim 2\theta = 1.5$ measured at room temperature remained almost unchanged up to 150°C and began to disappear at 175°C corresponding with the loss of secondary structure shown by the CD measurements in Fig. 4.

The preservation of the secondary structure of rhodopsin in L-B film does not necessarily mean the preservation of its functionality such as its ability to absorb photons in the visible range. To obtain spectrophotometric information, the preparation and the deposition of rhodopsin L-B film was performed under dim red light. The UV visible spectrum of 90 layers of rhodopsin was recorded in the dark at different

TABLE 2 Temperature dependence of rhodopsin secondary structure in solution

Temperature (°C)	α-Helices (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
25	68	12	9	11
45	63	12	9	16
65	59	14	10	17
85	42	24	14	20
95	40	20	13	27

Percentage of α-helix, β-sheet, β-turn, and random coil calculated from CD spectra of rhodopsin solution at different temperatures.

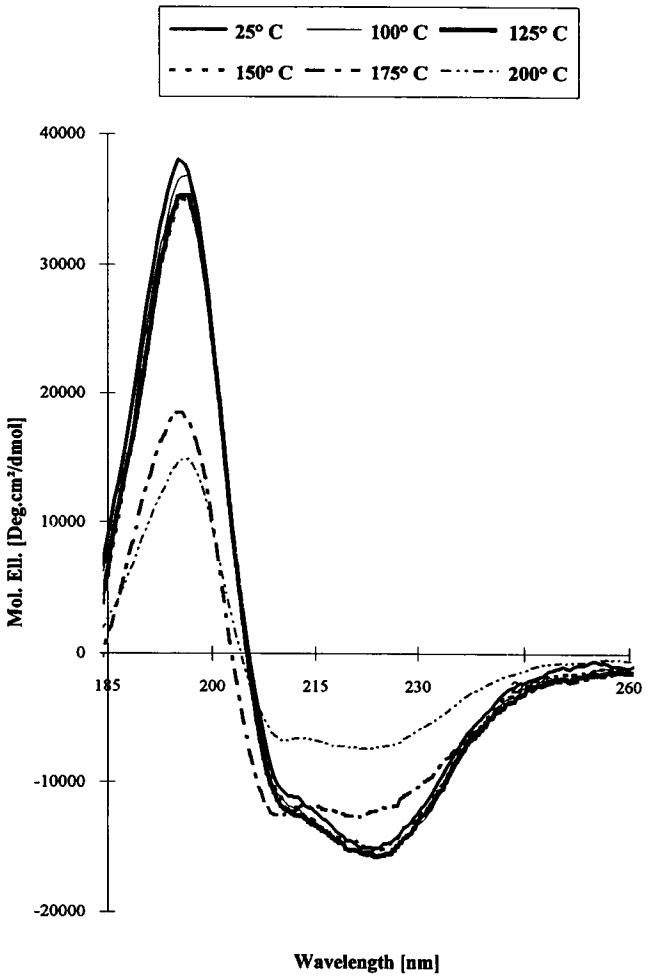

FIGURE 4 CD spectra of opsin L-B film at different temperatures. The sample (30 layers) was heated for 10 min in a Peltier-type thermostatic holder at the indicated temperature up to 110°C. For higher temperatures the sample was heated in an oven for 10 min and then measured for CD at 110°C.

temperatures. The curves show that the characteristic absorbance at 500 nm was preserved up to at least 80°C (Fig. 8 *c*) and began to have a small decrease at 90°C (Fig. 8 *d*). The increase of temperature was accompanied by an increase of light scattering. However, the absorbance maximum at 500 nm was still present at 100°C and the recorded spectrum remained identical when the sample was kept in room light for hours, showing no bleaching process. The same rhodopsin solution used to make the L-B film showed instead a collapse of the 500-nm absorbance maximum at ~70°C in the dark (data not shown), as already reported in the literature from calorimetric measurements on rhodopsin in disk membranes (Khan et al., 1991).

DISCUSSION

L-B films were made by spreading opsin solubilized in 0.75% β-octylglucoside on the surface of the subphase in the L-B trough and then compressing them isothermically

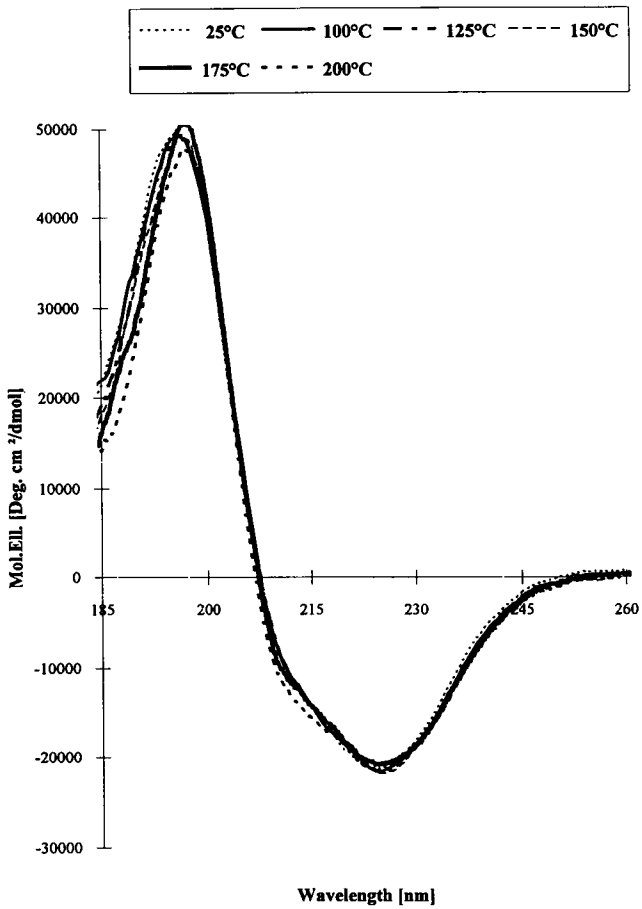

FIGURE 5 CD spectra of rhodopsin L-B film (30 layers) at different temperatures. The procedures for sample heating were the same as in Fig. 4. Rhodopsin L-B film was prepared in dim red light.

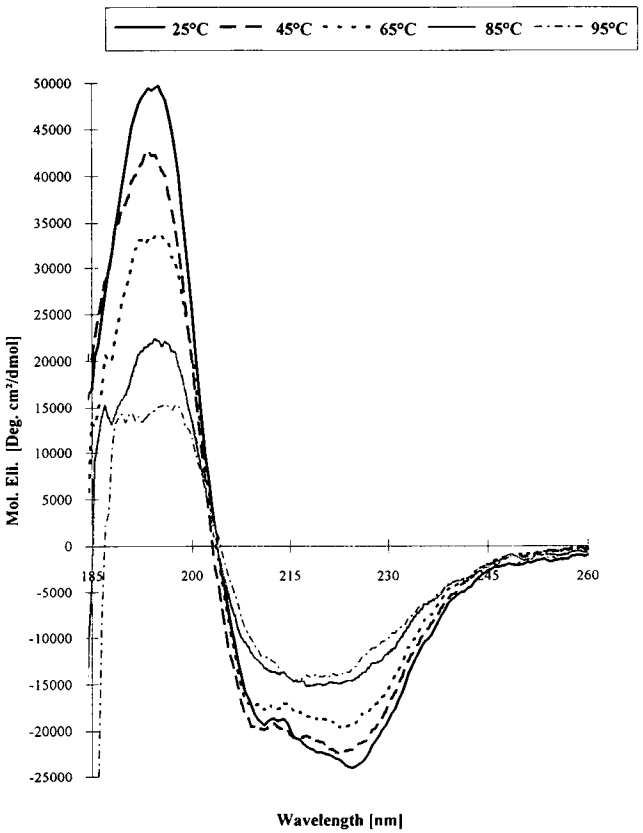

FIGURE 6 CD spectra of opsin solution at different temperatures. The sample was heated in a Peltier-type thermostatic holder at the indicated temperature for 10 min.

with two moving barriers. As reported in the literature (Lvov et al., 1991; Hann, 1990), it seems well established that the detergent molecules with short hydrocarbon chains, such as the β-octylglucoside used in these experiments, do not form stable monolayers at relatively high surface pressure, so that during compression they are transferred to the subphase in the form of micelles. Therefore, it is very likely that in these conditions a homogeneous film made up of mainly opsin was formed, with each molecule in close contact with the other, as suggested by the surface per molecule of ~1100 Å^2 deduced from nanogravimetric measurements. This value, which is affected by an error

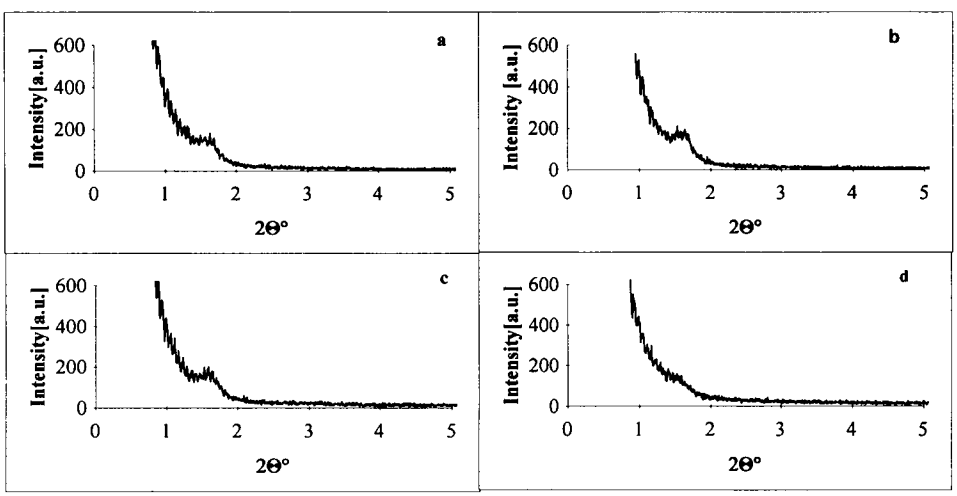

FIGURE 7 X-ray diffraction patterns of 30 layers of opsin L-B film at different temperatures: 25°C (*a*), 100°C (*b*), 150°C (*c*), and 175°C (*d*).

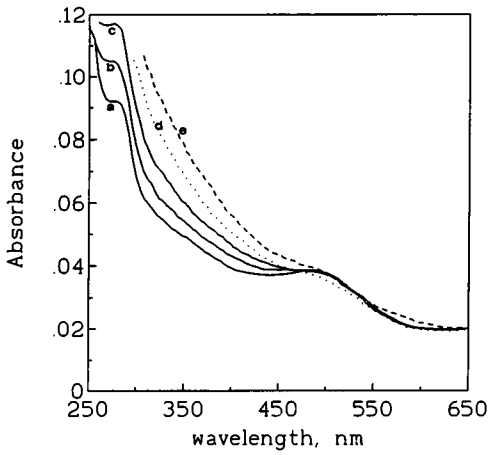

FIGURE 8 Absorbance spectra of rhodopsin L-B film (90 layers) at room temperature (*a*), 60°C (*b*), 80°C (*c*), 90°C (*d*), and 100°C (*e*). Rhodopsin film was prepared, deposited and dried in vacuum in dim red light, and then put in the oven for 10 min at the temperatures indicated. The spectrophotometric measurements were made at room temperature.

of ~20%, is in accordance with those reported for the area occupied by one molecule of rhodopsin without the surrounding lipids: ~800 Å^2 was obtained for frog rhodopsin (Dratz and Hargrave, 1983) and ~1100 Å^2 was deduced from the measurements of surface pressure on bovine rhodopsin films (Salesse et al., 1990). Furthermore, the opsin molecule in the L-B layer would have the longer cylindrical axis perpendicular to the plane of the film as in native disk membranes. This was suggested by the x-ray diffractometric measurements shown in Fig. 2, from which a periodicity of ~59 ± 3 Å can be deduced. This value is comparable with 60–65 Å as reported for the longer dimension of the rhodopsin molecule (Dratz and Hargrave, 1983). The same conclusion was reached by Salesse et al. (1990); that is, the orientation of bovine rhodopsin in films compressed at lateral pressure of 38 mN/m (very near the deposition pressure of 35 mN/m used in our experiments) was the same as in intact disk membranes. From this data a picture emerges of a two-dimensional L-B layer mainly made up of opsin molecules that are packed closer than in the already crowded disk membranes. In the latter ones, in fact, the molecules of rhodopsin per square millimeter are ~2.7 × 10^{10} (Stryer, 1986) and the disk membrane area per molecule occupied by one molecule of rhodopsin with the surrounding lipids was calculated to be ~3400 Å^2 (Dratz and Hargrave, 1983).

The CD spectra of opsin solutions were very similar to those of L-B films, suggesting that opsin did not alter its secondary structure significantly when assuming a two-dimensional quasi-crystalline configuration of the film.

The most striking property of rhodopsin in L-B films was the thermal stability of its secondary structure, which was maintained up to ~150°C in room light and up to at least 200°C in dim red light. This improvement of stability was also measured in disk membranes where the temperature of rhodopsin denaturation was ~56°C for bleached samples and ~72°C for samples kept in the dark (Khan et al., 1991). This is because of the more compact conformation of rhodopsin when it binds its chromophore compared with that of bleached rhodopsin in which the linkage between opsin and retinal is broken.

The thermal stability of opsin layers was also supported by x-ray diffraction measurements. The periodicity of ~59 Å was maintained almost unchanged up to 150°C, showing a good correlation with the negligible changes of the CD spectra in the same range of temperatures. At 175°C, however, the periodicity of the opsin layers was partially lost probably because of a kind of melting of the protein in correspondence with the change in the secondary structure shown by the CD spectra at the same temperature.

The increased thermal stability of opsin in L-B films seems to be a general property shared by other proteins (Nicolini et al., 1993; Erokhin et al., 1994; Shen et al., 1993) and is likely a result of the molecular order induced by the L-B film formation. In a CD study on anti-insulin antibody, a lyophilized sample was found more resistant to temperature than the corresponding antibody solution but less resistant than the same antibody in L-B films, leading to the conclusion that, although dehydratation plays an important role in structure preservation, the main stabilizing factor is the molecular close packing of the L-B film (Erokhin et al., 1994; Nicolini, 1995).

The characteristic absorbance maximum at 500 nm,on the other hand was less stable, as it began to decrease between 90 and 100°C. When exposed to light, rhodopsin in L-B films did not bleach, as expected, because the binding between retinal and opsin does not hydrolyze in dry conditions. After flash illumination at room temperature, rhodopsin embedded in dry gelatin films (Wald et al., 1950) or in air-dried phospholipid multilayers (Applebury et al., 1974; Korenbrot and Pramik, 1977) was converted into thermally stable metarhodopsin I (absorbance maximum at 480 nm). In our experiment, however (see Fig. 8), the exposure of rhodopsin L-B films to the room light apparently did not affect the 500-nm absorbance maximum. Therefore, this result could be interpreted as if rhodopsin had reached a kind of photostability when assembled in L-B films, unless rhodopsin was converted into its intermediate lumirhodopsin, which has a maximal absorbance at ~497 nm. The latter is so close to the absorbance maximum of rhodopsin as to be indistinguishable in our experimental conditions. This last interpretation would be in accordance with the results reported by Guérette et al. (1988) on the photochemical activity of the dried, aggregated form of bovine rhodopsin depleted of detergent. In conditions that share some similarities with ours, these authors found that the bleaching sequence of rhodopsin when exposed to daylight reached the step of lumirhodopsin, which then remained stable at room temperature.

The increase in light scattering of rhodopsin films with the temperature (see Fig. 8) was unexpected. It might have been a result of some degree of aggregation of rhodopsin molecules during heating.

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