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Optical and electrical properties of bacteriorhodopsin Langmuir–Blodgett films

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Abstract

Langmuir–Blodgett films prepared from purple membranes dissolved in pure hexane were characterized for M-state decay kinetics and photocurrent production. These studies indicated that the films are preferentially oriented with the cytoplasmic side of the *bacteriorhodopsin molecule facing away from the surface of the tin oxide coated glass electrodes*. Characterization of Langmuir–Blodgett films in high salt strongly indicates that the transient photocurrents observed are directly due to proton release and uptake and not to charge separation. Published by Elsevier Science S.A.

Keywords: Bacteriorhodopsin; Langmuir–Blodgett films; Opto-electrical properties

1. Introduction

Bacteriorhodopsin (bR) is a light-sensitive protein produced by the bacterium *Halobacterium salinarium*. This transmembrane protein is located in a structurally distinct area of the plasma membrane known as 'purple membrane' [1]. When this protein is activated by yellow light, it undergoes a photocycle whereby a proton is transported from the cytoplasmic side of the membrane to the periplasmic space. Retinal, the chromophore that is bound to Lys-216 of the protein by a protonated Schiff-base, is responsible for the absorption that occurs at the maximum of approximately 570 nm. A quantum of yellow light induces a *trans–cis* isomerization of the retinal, followed by transitions of the protein–retinal complex through a number of intermediate states (I, J, K, L, M, N, and O) that are characterized by different protonation states of the Schiff base and of several amino acid residues within the protein [2,3]. The M-state of the photocycle is formed when, as a result of isomerization of retinal, the proton on the Schiff base is transferred to Asp-85, and subsequently a proton from another amino acid, possibly Arg-82, is released into the solution on the periplasmic side of the membrane [4]. Reprotonation of the Schiff base is from the Asp-96 that is reprotonated from the cytoplasmic solution. The bR, like other membrane-bound proteins normally operates at an interface between high dielectric (water) and low dielectric (membrane) phases. The vectorial nature of the proton transport demands that the protein in the purple membrane be oriented at this interface. Hwang et al. [5] prepared Langmuir–Blodgett (L–B) films containing bR membrane fragments by combining the bR purple membrane fragments with soybean phosphatidylcholine (soy PC). These L–B films were examined by adsorption spectroscopy and electron microscopy using shadowing and freeze fracturing techniques. In addition, surface pressure and surface potential vs. area isotherms of the purple membrane interface films using 7:1 bR/soy PC monolayer indicated the presence of a net negative charge in the films or the introduction of permanent dipoles or both. Since bR has a large permanent dipole [6], this could contribute to the change in the observed potential, if the molecules were oriented at the interface. Using electron microscopy, it was observed that the purple membrane fragments were not completely dissolved in the hexane solvent used to prepare the films, but randomly distributed and separated by smooth spaces. The authors assumed that the smooth areas were occupied by soy PC and purple membrane lipids in a monolayer. Using freeze fracture preparations, they concluded that more than 85% of the purple membrane frag-

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ments were attached to the glass surface with the inner membrane (cytoplasmic side) toward the glass sub-aqueous phase.

Recently, it has been suggested [7] that L–B films prepared on indium–tin oxide modified optically transparent electrodes (ITO–OTE) were oriented with the cytoplasmic side oriented to the electrode surface. Their data, however suggests that the films are oriented cytoplasmic side up rather than down. These authors report that the displacement current caused by a dipole and/or proton transport in the bR molecule corresponds to a displacement of a positive charge toward the electrode surface and is inverse to the direction of proton transport [8]. This response is maximal when the cytoplasmic side is oriented to the electrode surface. The results obtained by Li et al. [7] cannot distinguish between displacement of a positive charge in the inverse direction or proton pumping. It is our belief that what is observed is indeed proton transport to the tin oxide electrode surface.

We have undertaken to examine the opto-electrical and kinetic properties of these films in more detail using the wild-type (WT) bR. We have also attempted to carry out experiments to distinguish between proton release and charge displacement by performing experiments in high salt concentrations where the effect of charge displacement should be minimized.

2. Materials and methods

2.1. Preparation of Langmuir–Blodgett films

Langmuir–Blodgett films were prepared as previously described [9] by dissolving the purple membrane fragments in reagent grade hexane. The films were all prepared on a Lauda MGW Filmwaag Balance. Millig water was used as the subphase and maintained at a constant temperature of 30°C. The ATO slides were pretreated with Malinkrodt Chem-Solv from Malinkrodt Chemicals (Paris, KY), water rinsed and then lowered into the subphase prior to spreading of the bR solution. The bR spreading solution was prepared by adding approximately 1 mg of bR (from 4–5 mg/ml water stock solutions) to 2 ml of hexane. The mixtures were then sonicated for approximately 1 min to get a bR/hexane suspension. This entire spreading solution was then dropped onto the water surface using a glass pipet. The material was left to stand for approximately 10 min before compression. Films were then compressed to an annealing target pressure of 20 mN/m. In all cases the films were left to anneal for at least 20 min until constant pressure was maintained. The ATO slides were then slowly withdrawn from the subsphase at a speed of 10 mm/min and then left to completely dry before characterization. Since there were no lipids added to these spreading solutions, much of the bR went into the subphase. However, the bR that did remain on the water surface formed a very steep compression curve and was found to be very stable, since films could be held at constant pressure for hours with little observable loss of area. In each case, when the ATO slide was withdrawn from the subphase, the barrier slowly compressed indicating direct transfer of the bR from the water onto the ATO glass electrodes.

Two batches of L–B films were prepared. The first batch was prepared by applying the spreading solution and then withdrawing the electrode through the stabilized purple membrane film to produce a single monolayer. The second batch was a multilayered film prepared by repeatedly lowering and withdrawing the same electrode through the Langmuir trough for a total of nine layers. The final films were air dried and stored under refrigeration.

2.2. Electrochemical set-up

The electrode set-up has been previously described in detail [10] and consisted of a flow-through system prepared from a electrochemical cell (BAS CC-4 Flow Cell) modified to hold the working tin oxide electrode. The metal body of the electrode served as the counter electrode and a silver/silver chloride electrode as the reference. The electrochemical cell cover was of black nylon with a hole 5 mm in diameter directly over the L–B film. The spot was illuminated with a mercury-arc lamp (LEP HBO 100). The cell was connected to a potentiostat consisting of an operational amplifier (OPA222) with a 1 MΩ resistor in the feedback circuit. The voltage across the resistor was amplified by an instrumentation amplifier (AD524) with gain = 1. The entire cell was shielded by placement in an aluminum box. The output of the electrochemical cell was connected to an oscilloscope (Tektronix TDS 410) capable of giving a response time for the system of approximately 4 ms. The polarity of the signal was adjusted so that a pH decrease caused an increase in observed current while increased pH produced a decrease in observed current in the downward direction. The photocurrents were determined at pH 7.0 and 3.0 in 0.005 mol/L phosphate buffer, 0.05 mol/L KCl. For high salt the KCl was increased to 0.5 mol/L.

2.3. Spectroscopy

Spectrophotometry was carried out on an on a diode array spectrophotometer (HP-8452A). The films were irradiated with 25 mW/cm^2 of yellow light from a 300 W halogen Kodak projector lamp through a 530 nm long-pass filter (Oriel 59500). The L–B films were examined spectrophotometrically in the dry state.

3. Results

Spectrophotometric examination of the 'single monolayer' L–B dried film in light and dark showed the presence of a photocycle as indicated by the small difference in the absorption spectra produced under these conditions.

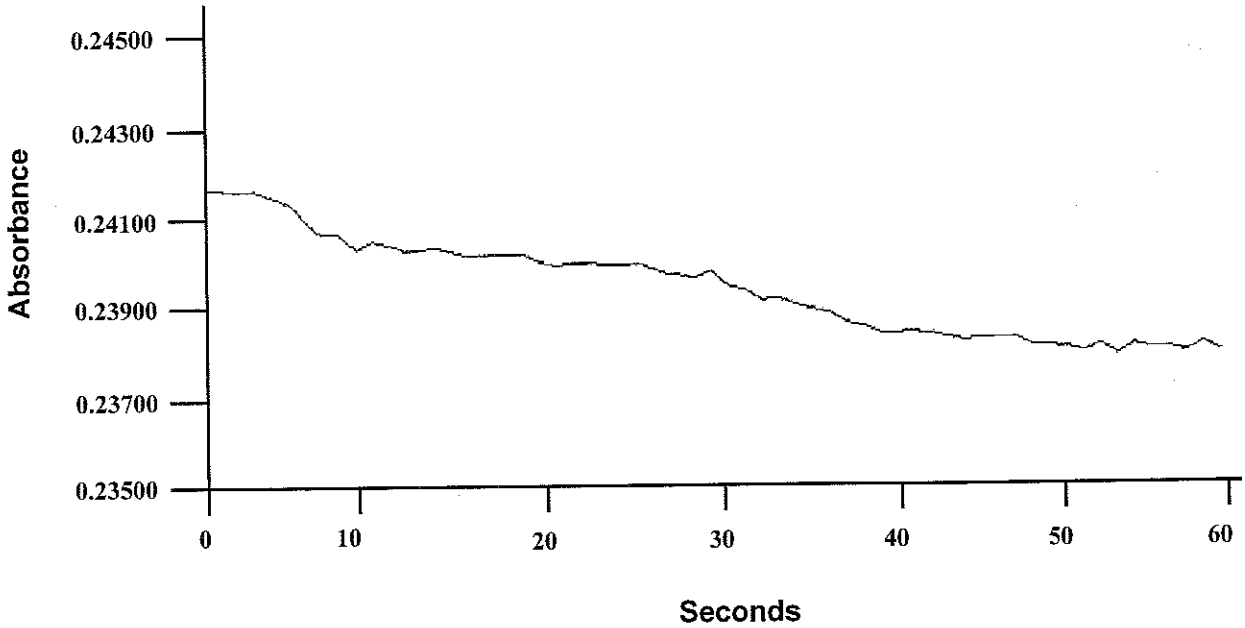

Fig. 1. Kinetics of M-state decay of dry multilayer Langmuir–Blodgett film monitored at 410 nm in the dark after 60 s of yellow light.

The spectra, however, did not show specific peaks at the expected wavelengths of 570 nm and 410 nm, but showed a difference across the entire wavelength range examined. Attempts to determine the kinetic half-times ($T_{1/2}$) for the conversion of the M-state intermediate to the ground state were unsuccessful with the 'monolayer' L–B films. Kinetics studies on the nine-layer sample (Fig. 1) monitored at 410 nm using yellow light for excitation gave a $T_{1/2}$

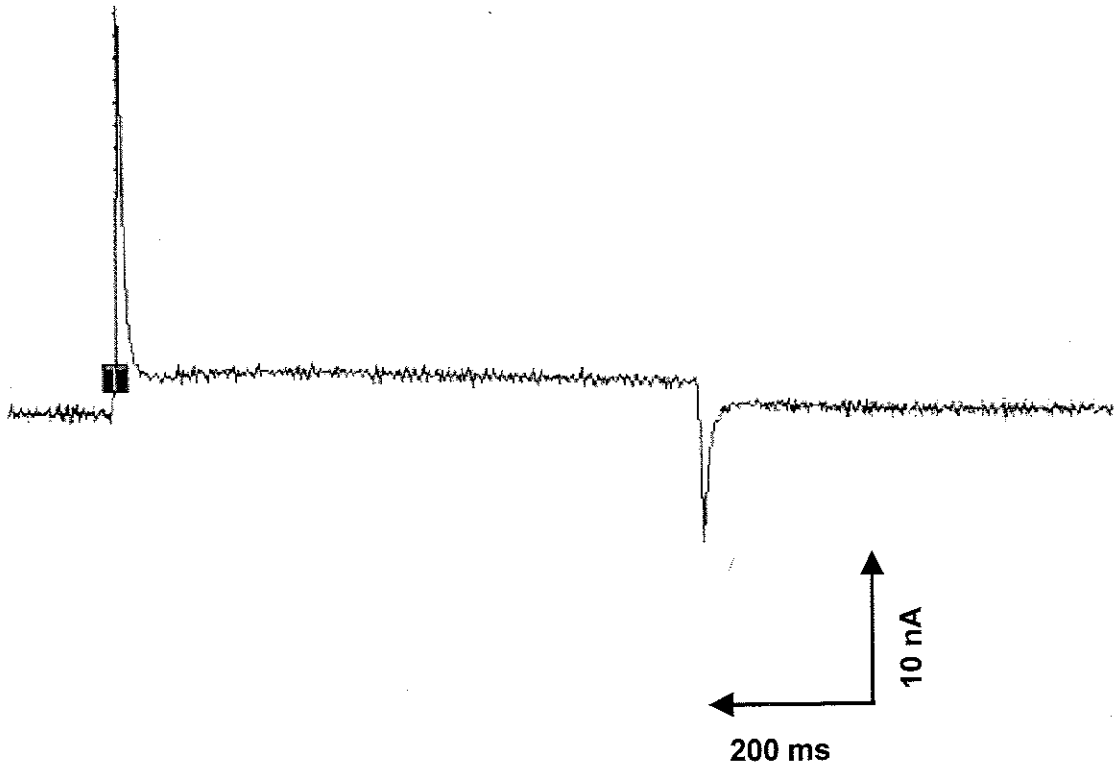

Fig. 2. Photocurrent transients of the 'monolayer' Langmuir–Blodgett film in contact with 0.005 mol/l potassium phosphate, 0.05 mol/l KCl at pH 7.0. The yellow light was maintained for 1 s. The initial response represents the light-on transient (pH decrease). The light-off transient is in the opposite direction (pH increase).

values for the M-state decay rates of 1.35 s and 5.40 s for the first and second exponential using a two exponential decay model [11]. Repeats of this same experiment on the same electrode gave very poor reproducibility. The data was considered only as proof that the L–B films containing the bR were active.

The transient photocurrents produced by the 'single monolayer' and the nine-layer L–B films at pH 7.0 are shown in Figs. 2 and 3 respectively. The transient light-on photocurrents are in the direction of pH decrease (proton release) while the light-off transients after a 1-s light exposure are in the opposite direction (proton uptake). The nine-layer films produced a maximum photocurrent of 80 nA ± 6 nA while the 'monolayer' film produced a smaller current of 37 nA ± 4 nA. The experiments were repeated at pH 3.0 (Figs. 4 and 5) with the observed photocurrent transients now in the opposite direction and smaller in size. Increasing the salt concentration to 0.5 mol/L at pH 7.0 with the 'monolayer' L–B film had no effect on the size or shape of the signal.

4. Discussion

The results of the spectral and kinetic determinations on the dried L–B films indicates that they are active even though we were unable to obtain an M-state decay rate for the 'monolayer' film. The spectra of the 'monolayer' L–B films lacked specific features but showed a difference between the sample exposed to yellow light vs. the sample in the dark. These spectra did not show any maxima at 410 nm or 570 nm. Since the 'monolayer' was prepared with the hydrophilic side facing the electrode surface, it is very unlikely that it is a true monolayer. Since the lipophilic side of the film would be facing the aqueous solution, it is possible that the film has folded into, at least, a bilayer or possibly liposome like fragments. The nine-layer L–B films on the tin oxide electrode is made of multiple layers with the last layer facing the aqueous phase being hydrophobic in nature. The spectrum of the nine-layer sample was similar to the 'monolayer', again suggesting that some readjustment may occur when the bR L–B films are placed

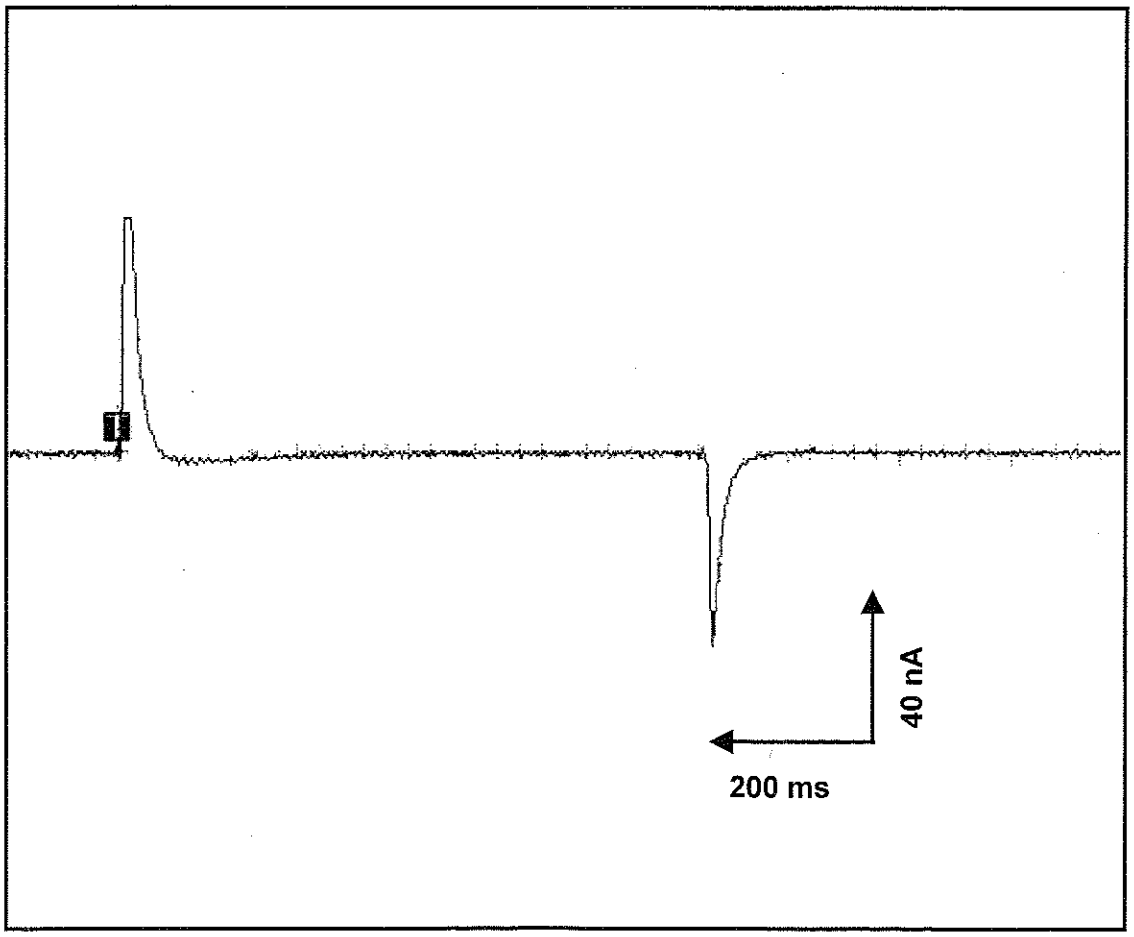

Fig. 3. Photocurrent transients of the multilayer Langmuir–Blodgett film in contact with 0.005 mol/l potassium phosphate, 0.05 mol/l KCl at pH 7.0. The yellow light was maintained for 1 s. The initial response represents the light-on transient (pH decrease). The light-off transient is in the opposite direction (pH increase).

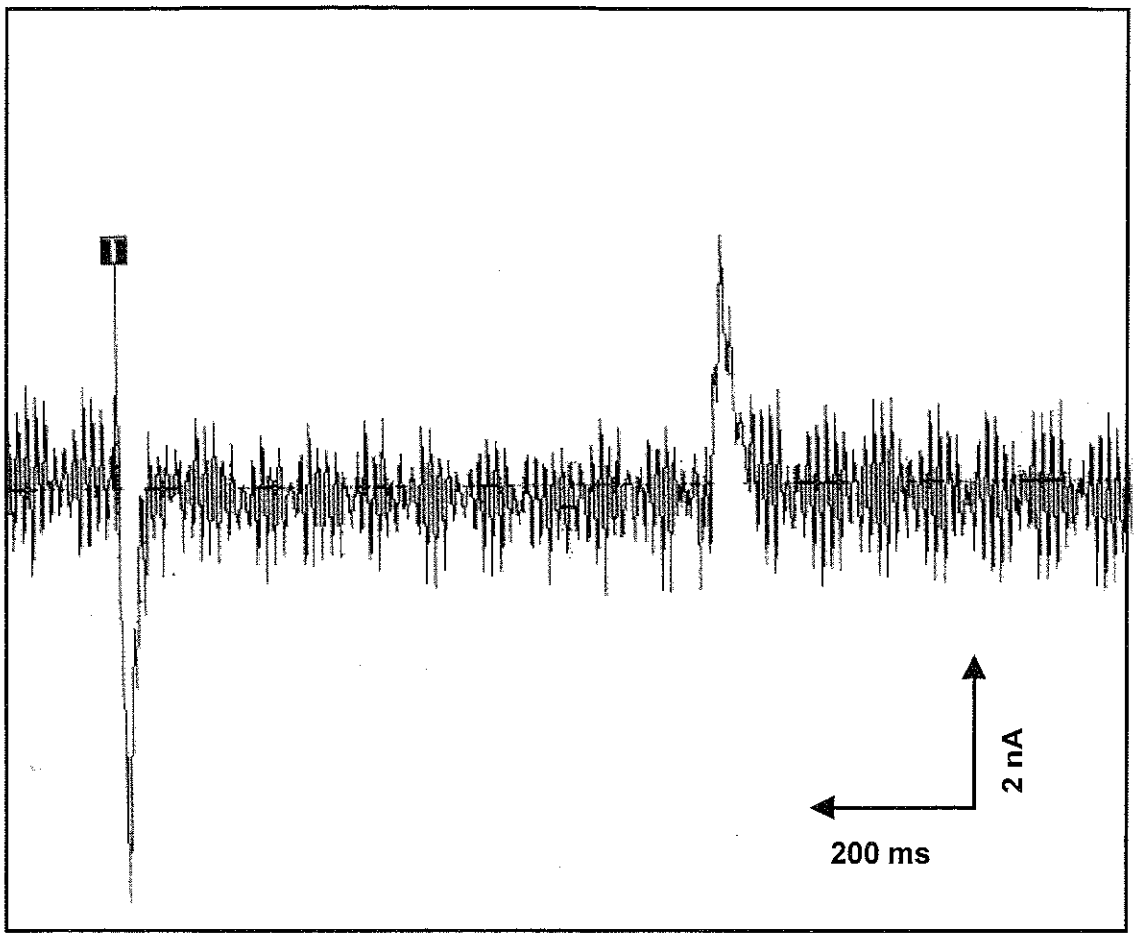

Fig. 4. Photocurrent transients of the 'monolayer' Langmuir–Blodgett film in contact with 0.005 mol/l potassium phosphate, 0.05 mol/l KCl at pH 3.0. The yellow light was maintained for 1 s. The initial response represents the light-on transient followed by the light-off transient. Both photocurrent transients are in the opposite direction from the photocurrent transients observed at pH 7.0.

in contact with buffer. When examined by difference spectra, it did show the expected peaks, although with a great deal of scatter. The M-state decay rate of the nine-layer film was observable (Fig. 1) yielding a half-times ($T_{1/2}$) within the general range of values observed in gelatin films [11]. The gelatin films, as with the L–B films, showed slowed M-state decay rates.

It is believed that the M-state decay rates are greatly affected by the limited water content and the low proton mobility. Water is necessary inside the protein for the reprotonation of the Schiff base because the largest part of the barrier is the enthalpy of separating the proton from Asp-96 (approximately 40 kJ/mol in the wild-type bR) [12]. In the case of the gelatin films, the presence of the polymer binder in the matrix of the film resulted in additional slowdown of the bR cycling activity. In the case of the L–B films, the result is rather surprising, in that one would expect the presence of the water opposite the film would permit sufficient hydration since the L–B film more closely resembles a natural membrane. Hwang et al. [5], indicated that the kinetics of decay of the intermediate are slower than in aqueous suspensions of purple membrane. Although the authors gave no values for the M-state decay rate, the curve they presented, monitored at 410 nm over a 3.0 s time span, showed slowed M-state decay. The figure shows the M-state decay still decreasing at 3.0 s, when the experiment ended. This data suggests that the bR is not fully hydrated in the L–B films even in the presence of a large excess of water.

The observed light-on photocurrent transients of our 'monolayer' and the multilayer bR–ATO films showed peak currents of 37 nA and 80 nA respectively. These data also suggest that the transient photocurrent is due to proton release. It is very likely that the multilayered L–B film tiled and filled in spaces on the ATO surface that would have been left bare on the 'monolayer'. Since the difference between the nine-layered L–B film and the single-layered L–B films is less then a factor of 3, it appears that only the bR next to the ATO is producing the transient photocurrent.

The photocurrent transients observed in our L–B films were similar to those observed by Robertson and Lukashev [13] using electrophoretically deposited oriented films on ATO. These electrodes show that the transient opto-electri-

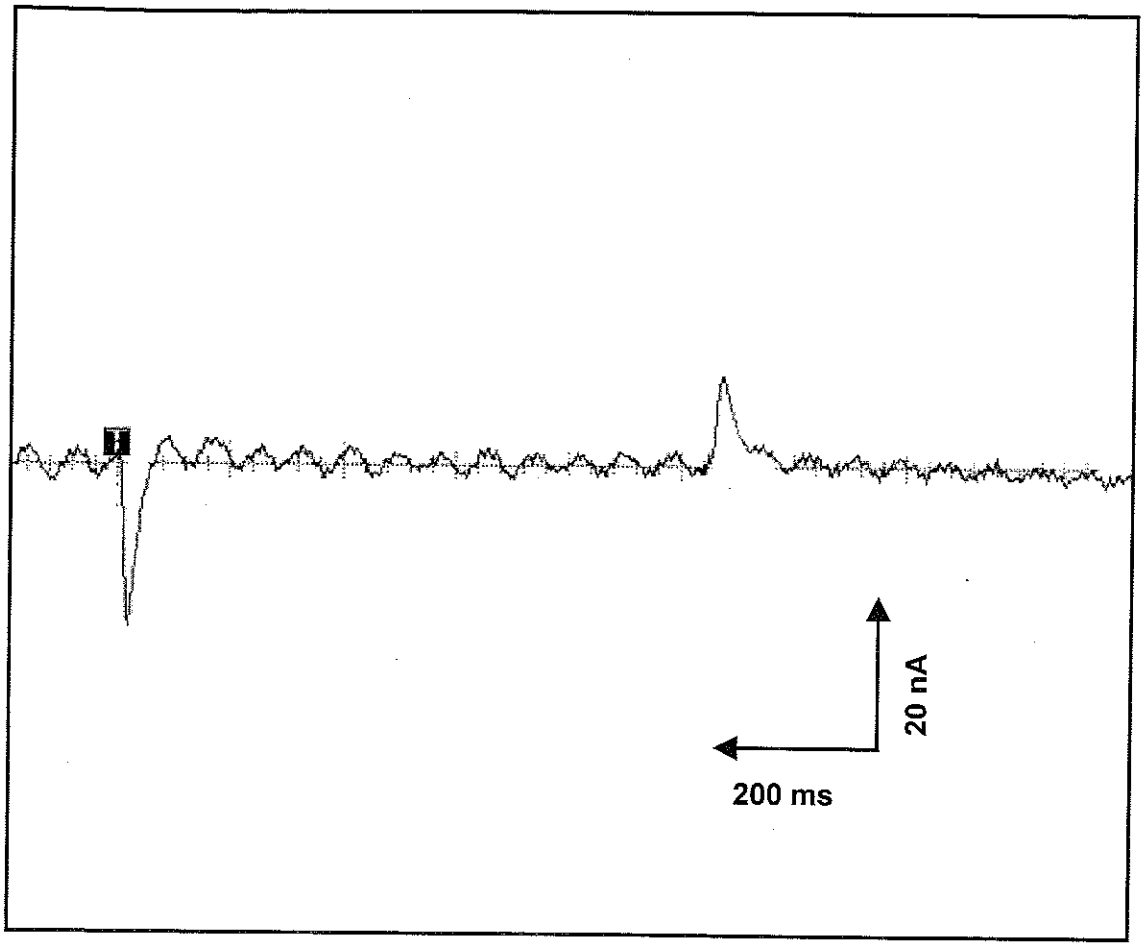

Fig. 5. Photocurrent transients of the multilayer Langmuir–Blodgett film in contact with 0.005 mol/l potassium phosphate, 0.05 mol/l KCl at pH 3.0. The yellow light was maintained for 1 s. The initial response represents the light-on transient followed by the light-off transient. Both photocurrent transients are in the opposite direction from the photocurrent transients observed at pH 7.0. Each box in the X direction represents 200 ms. Each box in the Y direction represents 20 nA.

cal response observed on irradiation with yellow light is due to the change in pH at the electrode surface caused by proton release. Antimony was substituted for indium because the ITO electrodes responded to yellow light producing a current. Responses similar to those observed in the bR–ATO electrodes were generated by introduction of dilute HCl using a flow-injection system with the bare ATO electrodes. These data strongly suggest that the photocurrent responses observed with irradiation is caused by proton release at the surface of the electrode, not uptake. Removal of the visible light source on the film gives a reverse signal. This is due to an increase in pH at the electrode surface, representing proton uptake. Additional experiments showed that by changing the prevailing pH from 7.0 to 3.0, one observed a reversal in the direction of the opto-electrical response on irradiation, indicating proton uptake. This occurs because the mechanism involved in proton release is shut down or greatly slowed by the decreased pH. It is reasonable to assume that the pKa of the amino acids involved in release (probably at the Arg-82 and/or Asp-85 positions) remains protonated at low pH and therefore does not release a proton to the external environment or releases the proton at a rate that our instrumentation cannot detect. However, on removal of the light source, detectable uptake does occur and protons are removed from the surface of the electrode. These data strongly suggests that there may be some misunderstanding of the experimental data previously observed [7,8]. It is also possible that the addition of the soy PC to the ITO-preparation induced results unlike those observed by Robertson and Lukashev [13] on ATO electrodes using electrophoretically deposited oriented purple patches and by Weetall et al. [10] in a more detailed study of the effects of pH on photocurrent produced in ATO electrodes.

This study was undertaken to re-examine this question using L–B films prepared without the addition of soy PC. For this study, the purple membrane has been suspended in pure hexane, without the addition of any phospholipid, for preparation of the bR Langmuir–Blodgett films. Their data strongly suggests that the films are oriented with the cytoplasmic side of the membrane away from the surface of the electrode.

Another factor that must be considered is the difference in the tin oxide electrodes. We observed that ITO gave transient photocurrent when irradiated with yellow light, while ATO showed no response under identical conditions. The differences in the nature of these two electrode types may also play a role in the observed differences between our results and the previously data.

Data collected on these L–B films under acid conditions showed no release or very slowed release of protons at a rate undetectable by our instrumentation. It did not prevent proton uptake that was observed on irradiation with yellow light (Figs. 4 and 5). The transient photocurrent uptake signals, under acid conditions, were decreased by approximately one third, suggesting that, at least, two thirds of the bR molecules are oriented with the cytoplasmic side away from the electrode surface.

The addition of 0.5 mol/l KCl to the system did not effect the photocurrent transients in any way. This strongly suggests that the observed photocurrent transients are due to proton release and uptake rather than charge separation. The high salt concentration should eliminate any observable signal due to charge separation within the bR L–B film. It is possible that charge separation contributes to the overall response to irradiation. However, this contribution is too small or too slow for our system to detect.

5. Conclusions

The data presented here argues that the bR orientation on a ATO electrode prepared under those conditions is predominantly with the cytoplasmic side up rather than down. As prepared, one would expect the hydrophilic side of the membrane to be next to the surface. However, it is most unlikely that the hydrophobic portion of the monolayer would remain in the presence of water in the photochemical cell. It is possible that the L–B film has reoriented into a bilayer or multilayer structure with the hydrophilic portion facing out rather than toward the electrode surface. It is also possible that we are observing only that portion of the bR oriented cytoplasmic side up. However, it this case, one would expect to observe a larger proton uptake signal. We did not observe such a signal. In addition, the experiment in 0.5 mol/l KCl, strongly suggests that the transient photocurrent observed on irradiation with yellow light is due entirely to a pH decrease at the surface of the electrode caused by proton transport to the electrode surface. If there is a photoelectrical effect due to charge separation, it is either too fast for our system to detect or it is below the detection limits of our instrumentation. We must also consider the possibility that some signal is generated by the use of ITO electrodes that do respond to yellow light.

We believe, that the orientation of our bR L–B films are not as previously described [5]. Unlike the L–B films described by Hwang et al. [5], we did not add any soy PC. It is possible that by not adding any soy PC, our films are not directly comparable. The authors state in their report, that more than 85% of fracture faces appear rough, that is, corresponding to the inner membrane half. Since the freeze fracture is that of the membrane half attached to the glass support, the electron micrographs indicate that, in the interface film, over 85% of the purple membrane fragments are oriented with their cytoplasmic surface towards the aqueous subphase. Since the freeze fracture data were generated on fresh L–B films, it is possible that they were indeed as described. However, when placed in an aqueous environment, where the lipophilic side would face the aqueous phase, they could reform into bilayers or multilayers with the cytoplasmic side away from the electrode surface. This could explain both our data and the previously published data [5,7].

In order to prove this hypothesis, we plan to carry out further experiments using the mutant bR S35C. This mutant has a cysteine substituted in one of the loops, outside of the lipid bilayer. By placing a reporter group on the cysteine we should be able to determine its location after we prepare the L–B film and determine whether the film changes configuration after re-exposure to water.

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References

[1] D. Oesterhelt, W. Stoeckenius, Nature New Biol. 233 (1971) 149.
[2] T.G. Ebrey, in: M.B. Jackson (Ed.), Thermodynamics of Membrane Receptors and Channels, CRC Press, Cleveland, 1993, p. 352.
[3] R.A. Mathies, S.W. Lin, J.B. Ames, W.T. Pollard, Ann. Rev. Biophys. Biophys. Chem. 20 (1991) 491.
[4] S.P. Balashov, R. Govindjee, M. Kona, E. Imasheva, E. Lukashev, T.G. Ebrey, R.K. Crouch, D.R. Menick, Y. Feng, Biochemistry 32 (1993) 10331.
[5] S-B. Hwang, J.I. Korenbrot, W. Stoeckenius, J. Membr. Biol. 36 (1977) 115–135.
[6] A.N. Kriebel, A.C. Albrecht, J. Chem. Phys. 65 (1976) 4575.
[7] B-F. Li, J-R. Li, Y-C. Song, L. Jiang, Mater. Sci. Eng. C3 (1995) 219.
[8] K. Koyama, N. Yamaguchi, T. Miyasaka, Science 265 (1994) 762.
[9] J.I. Korenbrot, Method Enzymol. 88 (1982) 45.
[10] H.H. Weetall, A. Druzhko, A.R. de Lera, R. Alvarez, B. Robertson, in preparation.
[11] T. Dyukova, H.H. Weetall, B. Robertson, BioSystems 1997, in press.
[12] Y. Cao, G. Varo, M. Chang, B. Ni, R. Needleman, E. Lanyi, Biochemistry 30 (1993) 10972.
[13] B. Robertson, E.P. Lukashev, Biophys. J. 68 (1995) 1507.