ОПТИЧЕСКИЕ СВОЙСТВА МОНОМЕРНОГО БАКТЕРИОРОДОПСИНА ИММОБИЛИЗОВАННОГО В ЖЕЛАТИНОВЫХ ПЛЕНКАХ

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Пурпурные мембраны представляют собой мембранные фрагменты клеточной оболочки галофильного микроорганизма *Halobacterium salinarium*, содержащие гексагонально-упакованные монослои светочувствительного белка бактериородопсина. В данной статье представлены морфологические, спектральные и кинетические характеристики пурпурных мембран, солюбилизированных двумя неионными детергентами, Тритоном-Х-100 и октил-β-d-глюкозидом и иммобилизованных в желатиновые пленки имеющие потенциальное техническое применение.

Optical properties of monomeric bacteriorhodopsin immobilized in gelatin films

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Abstract. Purple membranes are membrane fragments of the cell envelope of the halophylic microorganism *Halobacterium salinarium* containing hexagonally packed monolayers of the photosensitive protein, bacteriorhodopsin. Here we present the morphological, spectral and kinetic characteristics of purple membranes solubilized with two non-ionic detergents, Triton X-100 and octyl-β-d-glucoside and embedded in gelatin films having a potentiality for various technical applications.

INTRODUCTION Bacteriorhodopsin (BR) from the extreme halophile, *Halobacterium salinarium* is a well-studied retinal-protein complex, which is similar to visual rhodopsin of mammalia. Since its

discovery 30 years ago, its structure and photochemical properties have been examined in great detail [1]. Since the functioning of BR is highly stable and highly reversible, this membrane protein is being investigated as a photochromic material for the use in information processing by conventional optical and holographic systems [2,3]. BR immobilized in a film of a transparent polymer is an easy-to-use form for various technical applications. Such films or blocks are being actively developed and studied in different laboratories [4-6]. Optical parameters of these films strongly depend on the BR molecules environment (chemicals, polymer nature, water content, etc) and external effects: temperature, pressure, electric field, and dehydration, etc [3,6]. In most of the strains of *H salinarium* the BR molecules are arranged in purple membranes (PMs), which are 0.5-1 μ fragments of a lipid-protein monolayer of hexagonally packed BR molecules. PMs are stable but can be solubilized by detergents into BR monomers. We wish to report a study on the effect of PM solubilization with two non-ionic detergents Triton X-100 and octyl-β-D-glucoside on the spectral and photochemical properties of gelatin-embedded BR.

MATERIALS AND METHODS PM fragments were isolated from the cells of *Halobacterium salinarium,* strain ET 1000 according to the published technique [7] and sonicated for a total of 4 min at 20 kHz in an ice bath.

Preparation of the films. PM solubilization was performed by adding aqueous solutions of non-ionic detergents Triton X-100 or octyl-β-D-glucoside. Detergents, dimethyldichlorosilane and 300 Bloom gelatin were commercial preparations from Sigma Chem.Co., St.Louis MO and were used without further purification. The final Triton concentrations when incubated with PMs were 0.4-1.3 %. The Triton/BR molar ratios were 10:1, 15:1, 20:1, 25:1 and 30:1. Octylglucoside was used at the following octylglucoside/BR molar ratios: 100:1, 150:1, 170:1, 210:1, 260:1, 280:1, 320:1 and 400:1. Appropriate amounts of 1.2 M octylglucoside or 20% Triton X-100 were added to a 7.23 10^{-4} M BR suspension and incubated for 28 h at + 5^{o}C with continuous stirring of the mixture. Next, 0.9 ml of 8% aqueous gelatin was added to each of 1-ml sample at 38^{o}C followed by 10 min of stirring. The mixture was then introduced between two 5x5-cm glass supports (with 800 μm spacers) and was allowed to gel

at +9°C. After 1hour the upper glass support (pre-treated with dimethyldichlorosilane) was removed. The samples were allowed to dry at +9°C at 10 -15% relative humidity.

Electron microscopy. The glass microscope slides with attached gelatin films containing the different solubilization preparations of PMs were fixed in aqueous 2.5 % glutaraldehyde in Coplin jars for 2 h at room temperature. The slides were washed in distilled water and post-fixed in aqueous osmium tetroxide for 2 h. After washing in distilled water the slides were dehydrated in a graded series of alcohols beginning at 40 % with increments of 10 % up to 100 % for 10 min each. The slides were then transferred to 100 % acetone. The gelatin films were impregnated with a 1:1 mixture of acetone and Spurr low viscosity resin followed by immersion overnight in the Spurr resin alone. Strips of the gelatin films were cut away from the glass slide and embedded in Spurr Low Viscosity embedding medium in flat embedding moulds. Three to four strips could be placed in parallel in a single mould. The resin was polymerized overnight at 70°C. The embedded blocks were mounted in a flat embedding holder in an LKB Ultratome III ultramicrotome and horizontal sections through the strips were cut with a diamond knife. The sections mounted on 300 mesh copper grids were stained with 1 % uranyl acetate and 1 % lead hydroxide. Specimens were examined in a Philips CM12 electron microscope operating at 80 kV and micrographs recorded at a nominal magnification of x 28,000.

Spectral and kinetic measurements. Spectral and kinetic measurements of the films at the absorption maxima of the ground and photoinduced states were performed on the HP 8452A Diode Array spectrophotometer. The samples were considered to be dark-adapted (DA) if kept in the dark for more than 24 h and light-adapted (LA), as defined by the maximum absorption $A_{\lambda max}$ observed after complete relaxation from the M-state after 1-min saturating exposure. The power density of the green light (Kodak slide Ektagraphic projector, model B-2; 300 W, 120 V bulb with a combination of Oriel 59494 and 58856 filters which cut-off light between 470 and 590 nm) was 22.5 mW/cm^2. This power density was saturating only for the solubilized samples with maximum detergent/BR ratios.

RESULTS The extent of solubilization and the number of monomeric BR molecules increased with increasing detergent con-

centrations as determined by electron microscopy. Complete PM solubilization was achieved at a Triton/BR molar ratio of 30:1 or octyl-β-D-glucoside/BR molar ratio of 320:1.

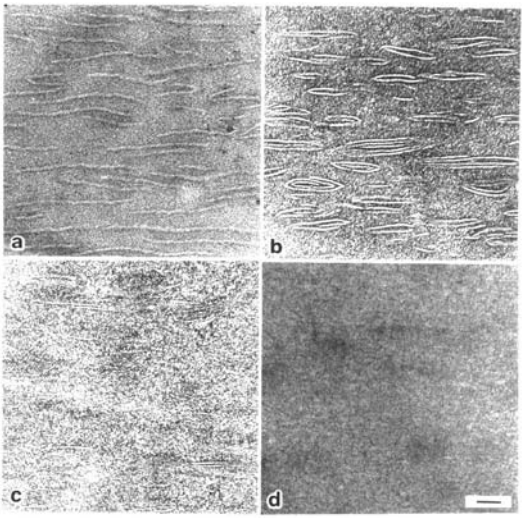

Figure 1. Electron micrographs of cross-sections of gelatin-embedded PMs solubilized with increasing amounts of octyl-β-D-glucoside: **a.** Control sample with native (unsolubilized) PM fragments; **b.** Octyl-β-D-glucoside/BR molar ratio of 100:1; **c.** Octyl-β-D-glucoside/BR molar ratio of 170:1; **d.** Octyl-β-D-glucoside/BR molar ratio of 400:1. The scale on **d.** equals to 500 nm.

Electron microscopy studies. Figure 1 shows electron micrographs of cross-sections of gelatin-embedded PM films. The PMs were solubilized with increasing amounts of octyl-β-D-glucoside. In the control sample, gelatin-embedded native PM fragments dehydrated on a glass support appear as flat membranes oriented in the plane of the support (a). Increasing amounts of octylglucoside caused twisting of PM fragments in a form of tubular structures (b) and progressive PM destruction (c and d). Together with tubular structures a few PM stacks were also found. Dehydration of the gelatin-embedded PM layers resulted in layer shrinkage (approximately 15-17 times) which, in turn, caused the tubular structures contract. Figure 1d shows complete PM solubilization. Similar results were obtained for Triton-treated samples.

Solubilization-induced spectral changes. Tables 1 and 2 present

experimental data on the optical parameters of LA and DA ground state and photoinduced M-state of BR in gelatin-embedded PM layers as influenced by the extent of PM solubilization. Both DA and LA BR samples showed a blue shift of initial absorption maxima, which was more significant for DA Triton-treated samples. It should be noted that the absorption peak maximum for the control LA BR sample is already blue-shifted by almost 10 nm (see Tab.1, 2). However, the LA maxima for the solubilized samples in aqueous suspension (according to the literature data) and in gelatin films agree (Tab.3). LA samples with monomeric BR show the same initial absorption band maximum at 556 nm for both detergents (Tab.1, 2). In the course of dark adaptation, the extent of blue shift varies depending on the type and concentration of the detergent. For octylglucoside-treated samples the blue shift due to the dark adaptation does not exceed 2 nm (Tab.1); for Triton-treated samples the shift can increase to 8 nm (Tab.2). The solubilization process also results in a blue shift of the M-state absorption maxima. The samples with monomeric BR (complete PM solubilization) exhibit maximum absorption at 400 nm. This shift has not been previously observed. The aqueous suspension of monomeric BR was shown to exhibit the wavelength maximum position for the M-state at 412 nm [8,9].

Table 1. Photoinduced optical parameters for octyl-β-D-glucoside-treated purple membranes embedded in gelatin film after 1 min of saturating light exposure at power density of yellow light 22.5 mW/cm^2. The samples are light-adapted.

OG/BR molar ratio	Ground state $\lambda_{max,}$ (DA), nm	Ground state $\lambda_{max,}$ (LA), nm	M-state $\lambda_{max,}$ nm	Total time for ground state recovery, min	Bleaching efficiency, $\Delta A_{BR556}/A_{556,}$%
Control	558	560	406	10	33.7
150:1	556	556	406	18	36.1
210:1	556	556	404	21	36.9
260:1	554	556	404	28	41.2
280:1	554	556	400	23	41.8
320:1	554	556	400	31	46.2

Table 2. Photoinduced optical parameters for Triton X-100-treated purple membranes embedded in gelatin film after 1 min of saturating light exposure at power density of yellow light 22.5 mW/cm^2. The samples are light-adapted.

Triton/BR molar ratio	Ground state $\lambda_{max,}$ (DA), nm	Ground state $\lambda_{max,}$ (LA), nm	M-state $\lambda_{max,}$ nm	Total time for ground state recovery, min	Bleaching efficiency, $\Delta A_{\lambda max}/A_{\lambda max,}$ %
Control	558	560	406	10	50.1

Triton/BR molar ratio	Ground state $\lambda_{max,}$ (DA), nm	Ground state $\lambda_{max,}$ (LA), nm	M-state $\lambda_{max,}$ nm	Total time for ground state recovery, min	Bleaching efficiency, $\Delta A_{\lambda max} / A_{\lambda max,}$ %
10:1	552	556	406	22	78.7
15:1	548	556	406	23	80.7
20:1	548	556	406	29	82.1
25:1	548	556	405	32	78.9
30:1	548	555	400	34	84.5

Table 3. The effect of solubilization on the absorption maxima of the ground state of light-adapted BR depending on the environment. *Data from (8).

Environment	Control sample, nm	Triton X-100, nm	Octyl-β-D-glucoside, nm
Aqueous PM suspension	568-570	555*	557*
Gelatin film	560	555	556

Solubilization-induced changes in the photocycle. We observed an increase in total time for ground state regeneration in the BR films in the presence of both detergents. This increase is 3-4 times that of the control (Tab.1, 2). However, the native (control) samples itself are known to exhibit the total time of 10 min for the ground state regeneration as compared to that of 10-50 ms for an PM aqueous suspension due to the dehydration of the matrix [6]. There is an evidence of up to 3-fold decrease in the half-time of M-state formation in an aqueous suspension of solubilized PMs [8]. We did not observe this effect at any detergent concentrations. Half-times of M-state formation were similar for all samples and averaged between 1.2 and 1.4 s (Tab. 4).

Table 4. Kinetic parameters of gelatin-embedded purple membrane samples solubilized with non-ionic detergents Triton X-100 and octyl -β-D-glucoside. Samples are light-adapted.

Detergent/BR molar ratio	Half-time of M rise at 400 nm, s	Half-time of M decay at 400 nm, s	Total time for ground state recovery, min
Control sample	1.4	4.0	10
octylglucoside/BR 320:1	1.4	6.5	31
Triton/BR 30:1	1.3	10.0	34

The data on the bleaching efficiency, the maximum absorbance changes at 556 nm are of the special interest. We define this parameter as the ratio of the photoinduced absorbance change at the maximum absorption of the initial state related to the quantity of the photoactive molecules at equal exposures ($\Delta A_{\lambda max} / A_{\lambda max}$). It is pro-

portional to the light intensity and the lifetime of the M-state. Bleaching efficiency values increased as the concentration of both detergents was increased (Tab.1, 2) and at maximum concentrations of octylglucoside and Triton X-100 amounted up to 50% and 90%, respectively.

A 10-15 % absorbance decrease was observed in the BR films over several days after sample preparation. This decrease was accompanied by the appearance of a new absorption band at 380 nm only in the samples having high detergent/BR molar ratios. Figure 2 presents the data for gelatin-embedded PMs solubilized with Triton X-100, which were measured immediately after preparation and after 10 days. The photochemical activity of BR molecules at higher Triton/BR molar ratios remained the same as can be observed from the bleaching efficiency data, $\Delta A_{556}/A_{556}$ in Figure 2. The process of decomposition seems to cease within several days after the sample preparation, after which the properties of BR films remain unchanged.

Figure 2. Absorbance changes vs. storage time for the gelatin-embedded PMs treated with increasing amounts of Triton X-100. Curves 1a,b represent absorbance amplitude as Triton/BR molar ratio increases: **a.** 1 day after the sample preparation (■) **b.** 10 days after the sample preparation (▲). Curves 2a,b present the extent of maximum bleaching at 556 nm as Triton/BR molar ratio increases: **a.** 1 day after the sample preparation (■) **b.** 10 days after the sample preparation (▲).

DISCUSSION As shown in literature, detergents effect BR as PM dissociation into the monomeric form of mixed BR-lipid-detergent micelles occurs [10], replacing native lipid surroundings of

BR; each micelle generally contains one BR molecule. At low detergent concentration formation of tubular structures was observed due to lipid and charge asymmetry between two PM surfaces (PM lipids are known to be solubilized more easily than PM proteins). At the highest octylglucoside/BR molar ratio of 400: 1 complete PM solubilization occured and absence of all structures was observed in the electron micrographs. We believe that the formation of mixed BR-lipid-detergent micelles occurs as previously reported. These micelles are not visible in the electron microscope. Formation of these structures (tubular structures and micelles) could have caused the distance change between the charged amino-acid groups of the BR helixes, which in turn, affect the charge environment of the chromophore. It is likely that the modification of the membrane shape and the change in the distances between the helical segments of the BR molecule which causes the modification in the mutual arrangement of charged groups within the chromophore center finally results, in addition to all other effects, in the change of the lifetime of the M-state.

Contrary to the dynamics of the primary events in BR which experince only slight influence upon solubilization [11], the last stages of the BR photocycle associated with conformation changes are known to be influenced more heavily by the detergents. It was previously observed that there is a 10-fold increase in the half-life time of the M-state in an aqueous suspension of solubilized PMs [8]. For the monomeric BR sample as compared to the contro, we observed no more than 2 to 3-fold increase of the half-life time of the M-state (Tab.4). The smaller M lifetime increase observed in our experiments is most likely related to the dehydration process in the gelatin matrix. The dehydration itself causes the retardation of the photocycle by 3 to 4 orders of magnitude, masking any smaller changes. In monomeric BR, the flexibility of the molecule is greater, due to a free energy of the protein molecule. This energy increase, resulting from the disruption of protein/ protein and protein/lipid interactions as the solubilization proceeds, gives rise to a further increase in the M-state lifetimes. However, it should be noticed that the disadvantage of this flexibility is a lesser stability of the BR molecule. It was recently shown that retinal binding and protein secondary structure is much less stable in solubilized monomeric BR [12]. Hexagonally

packed protein–lipid structure is known to be a stabilizer of the protein.

At the maximum Triton/BR and octylglucoside/BR molar ratios, when PM is expected to be fully solubilized and BR monomers are present, we still observed changes in kinetics and significant differences in optical parameters of these two monomeric BR samples (see two last lines of Tab.1, 2). It is an argument for the formation of new bonds and/or interactions inside the micelle of BR, detergent and lipid. We believe that these optical changes are most likely due to the lipid-detergent environment of the BR and the modifying effect of the detergent, rather than BR monomerization. In case of gelatin films, an interaction may take place between the gelatin and the BR molecules in the mixed detergent-lipid-BR micelles by the way of various secondary bonds like hydrogen bonds between amino acid residues of both gelatin and the BR. In addition, large areas of hydrophobic interactions may be formed.

The M-state accumulation increases the bleaching efficiency of the solubilized sample as compared to that of the control. As seen from Tab.1, 2, for Triton-treated samples, the bleaching efficiency is much higher as compared to that for the octylglucoside-treated samples; at maximum detergent concentrations this amounts to 90% and 50%, respectively. For Triton-treated sample incomplete transition into M-state may be partially explained by the heterogeneity of the samples and by the fact that excitation light also excites the M-state molecules inducing reverse transition into the ground state. It is known that up to 5% of the M-state-molecules can undergo photoinduced reverse transition. These molecules are always present in the initial BR form and do not participate in the M-state formation.

BR films similar to those described here may find application in the field of optical processing, for instance, for the storage and retrieval of optical information, e.g. holographic recording [13]. Since the diffraction efficiency is proportional to the square of the quantity of the BR molecules in the M-state, the extent of efficiency in these films strongly depends upon the number of BR molecules converted into M-state. Thus, PM solubilization seems to be a promising tool to optimize this parameter of BR films.

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