0273-2289/05/120/0121/$30.00

Effect of Dehydration on Photoinduced Transformation in Gelatin Films Made With 14-Fluoro Bacteriorhodopsin Derivatives

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Received January 20, 2004; Revised July 19, 2004; Accepted August 17, 2004

Abstract

Photoinduced transformation in gelatin films made with 14-fluoro bacteriorhodopsin derivatives, both wild-type (WT) and D96N mutant, were studied. Spectral and kinetic peculiarities for these two types of samples were compared over a wide range of relative humidity (9–92%). Analysis of the results considered two existing photoinduced processes that occur in suspensions and films of corresponding pigments. It was demonstrated that there is a range of humidity in which the performance of fluorine WT bacteriorhodopsin gelatin films may offer a technological advantage compared with fluorine D96N bacteriorhodopsin.

Index Entries: Photoinduced transformation; bacteriorhodopsin; all-*trans* retinal; relative humidity; gelatin films; red-shifted species.

Introduction

Over the past decade there have been numerous possible applications of photosensitive natural proteins for bioelectronics. The light-transducing

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proteins such as bacteriorhodopsin, visual rhodopsin, and photosynthetic reaction centers are appropriate examples of biomolecular systems that have been investigated for optoelectronic applications. The photochromic protein bacteriorhodopsin represents a class of biologic materials of considerable interest for a variety of technological applications because of its nonlinear optical properties, photoelectric activity, and light sensitivity *(1–3)*.

Bacteriorhodopsin is grown in the purple membrane of a halobacterium called *Halobacterium salinarium (4)*. Bacteriorhodopsin is a unique light-energy-transducing protein that has a remarkable feature of forming a hexagonal crystalline array of bacteriorhodopsin molecule trimers *(5)*. The crystalline structure causes a substantial increase in the bacteriorhodopsin's stability toward chemical and thermal degradation. The light-absorbing chromophore is all-*trans* retinal covalently bound to Lys-216 via a protonated Schiff base linkage ($-C_{15}H=NH-$) *(6)*. The absorption of light by bacteriorhodopsin leads from *trans* to *cis* isomerization of the $C_{13}=C_{14}$ bond of the all-*trans* retinal.

Therefore, on light excitation a bacteriorhodopsin molecule passes through a photocycle with several short-time intermediates to a metastable M-state; from there it thermally relaxes to the original B-state on a timescale of 10 ms *(1)* with associated spectral recovery. The time required for the photocycle (10 ms) is considered too short for cache-memory applications.

A variety of techniques that are suitable for optical applications have been used for preparation of samples containing bacteriorhodopsin: aquatic suspensions *(7)*, polymer matrices *(8,9)*, emulsion produced by electrosedimentation of purple membrane fragments *(10)*, and Langmuir-Blodgett films *(11,12)*. The use of polymer-based films produced by embedding bacteriorhodopsin into water-soluble polymers followed by dehydration allows one to increase the lifetime of the M-state by several orders of magnitude *(8,13)*. It is preferential to have bacteriorhodopsin in the form of a thin film, because it is easier to manipulate (Fig. 1). Using chemical supplements, particularly organic nitrogen-containing chemicals, during film preparation increases the lifetime of the M-state (a potential information storage time) to minutes *(14)*.

Within the past two decades, many chromophore analogs have been incorporated into the binding sites of bacteriorhodopsin. Observation of the resulting opsin shifts and/or photochemical characteristics has been analyzed in terms of chromophore-protein electrostatic and conformationally restrictive interactions *(15)*. Chromophore (retinal) replacement in the bacteriorhodopsin binding site with an artificial analog affects the spectral and kinetic properties of the bacteriorhodopsin. In addition, site-directed mutagenesis of specific residues in the apoprotein leads to novel characteristics. Use of these techniques together provides researchers with a range of novel chromoprotein complexes that possess photochromic characteristics different from native bacteriorhodopsin *(16)*. Study of bacteriorhodopsin suspension reconstructed with 4-keto retinal (4-keto bacteriorhodopsin)

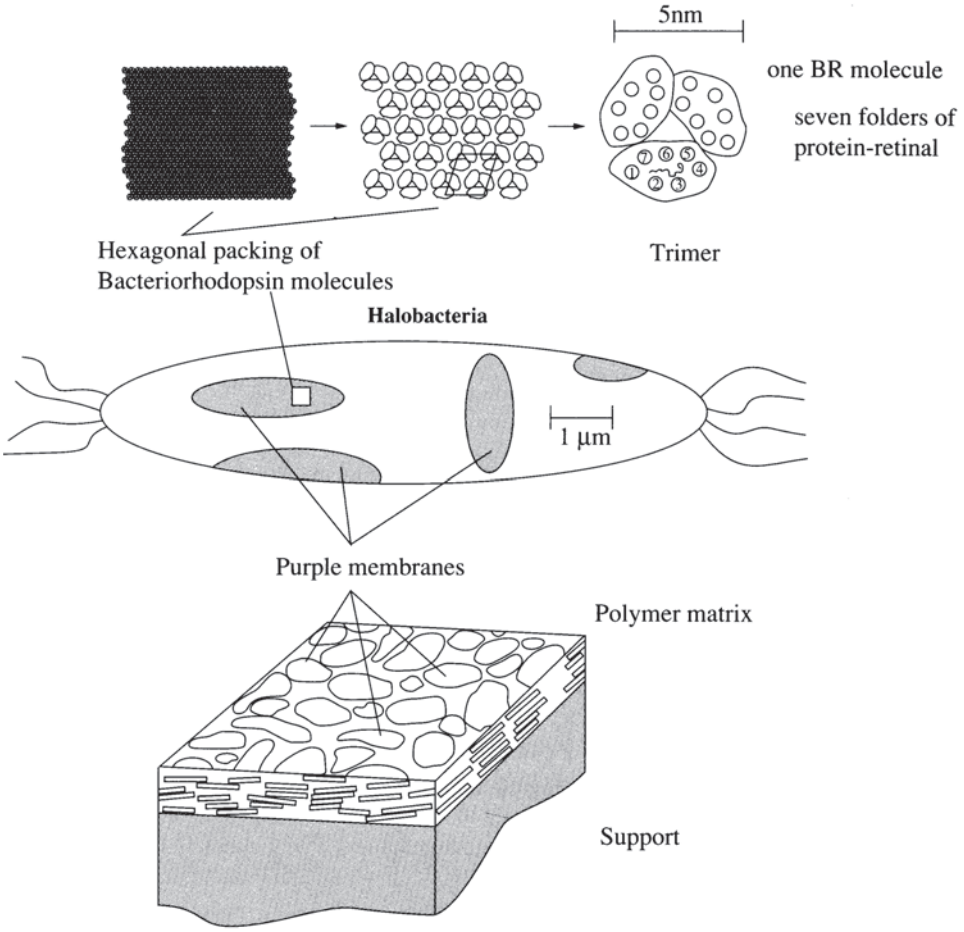

Fig. 1. Schematic illustration of bacteriorhodopsin packing in purple membrane and fabrication of polymer films.

has demonstrated photocycle reactions unlike the photocycle of native bacteriorhodopsin, which includes several spectrally and kinetically distinguishable M- and O-type intermediates *(17)*. Polyvinyl alcohol or gelatin films made with 4-keto bacteriorhodopsin show a decay time of M-state intermediate of not less than 30 min *(18)*. A combination of two approaches—specific replacement of the chromophore in some mutants and then comparison of photoinduced characteristics of 4-keto wild-type (WT) bacteriorhodopsin and 4-keto D96N bacteriorhodopsin—demonstrates an increase in the contribution of the most long-lived component of the M-state decay for the films based on 4-keto D96N compared with that for 4-keto WT *(19)*. This increase in the lifetime of M-state for films made with 4-keto D96N bacteriorhodopsin extends the operating range of the films and offers an advantage as a recording medium compared with the films made with 4-keto WT bacteriorhodopsin *(19)*.

Along with kinetic optimization, the use of chromophore analogs permits a shift in the bacteriorhodopsin absorption spectra while maintaining photochromic activity. Of particular interest are chromophore replacements that shift the initial absorbance to the red. This allows the use of small and inexpensive semiconductor lasers as a part of an optoelectronic device. An example of a red shift is seen in azulene derivative of retinal *(20)*. It is known that inserting strong electron-withdrawing halogen substituents at C_{13} or C_{14} of the chromopore polyene chain also leads to a red shift of the initial absorbance as well as to a greater photochromic shift (difference between initial absorption and M-state absorption). A greater photochromic shift results in a better image contrast. This suggests that an absorption maximum at a wavelength longer than 600 nm (i.e., red-shifted pigments) would be desired.

It was recently shown that 14-F retinal when incorporated into WT and D96N apomembranes, produced pigments with drastically different photoinduced behavior *(21)*. Previously, we studied the spectral and kinetic transformations of 14-F WT (bacteriorhodopsin) and 14-F D96N embedded in gelatin films. It was demonstrated that, unlike the study of water suspensions of the same pigments in which a red-shifted species at 660 nm was shown to be formed under the illumination in 14-F WT, but not in 14-F D96N, there were no drastic differences in photoinduced behavior between gelatin films based on 14-F WT and 14-F D96N *(22)*. We have not observed any photoinduced formation of red-shifted species at 660 nm for both types of films as opposed to the corresponding pigments in water suspension *(22)*. We suggested that a possible explanation of these facts arises from the kinetic characteristics of gelatin films that are different from those for water suspensions *(22)*. It is well known that the main factor in altering the kinetics of gelatin films is a variation in a hydration state by changing the environmental relative humidity (RH). Therefore, the next step in this study should be to vary the humidity of the fluorine bacteriorhodopsin gelatin samples in order to find the desired properties (large red shift). The investigation described herein serves to clarify this matter.

Materials and Methods

The activity of the apomembrane was tested by reconstitution with all-*trans* retinal (Sigma, St. Louis, MO). Both all-*trans* and 13-*cis* 14-F retinals were prepared as previously reported *(23)*, and then their isopropanol solutions were used for reconstruction procedure. The chemical additives diaminopropane (DAP) and guanidine hydrochloride (GuHCl) were of reagent grade (Sigma) and were added to the photosensitive mixture of the bacteriorhodopsin derivatives (14-F bacteriorhodopsin, both WT and D96N mutant) and gelatin binder.

The apomembrane was prepared by using photoinduced hydroxyl aminolysis: Bacteriorhodopsin suspension was illuminated ($\lambda > 500$ nm) in the presence of 0.5 *M* NH_2OH at pH 8.4. The sample was cooled to 7°C

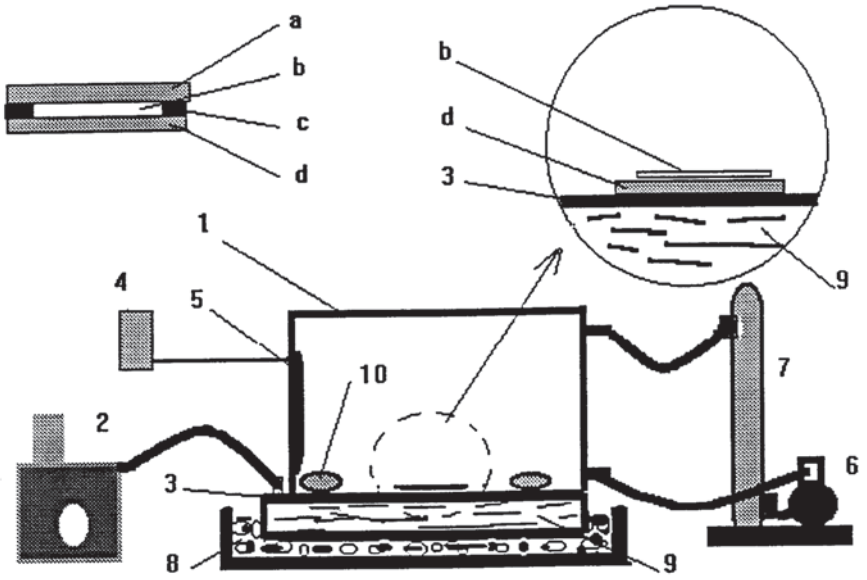

Fig. 2. Schematic view of a laboratory made setup for preparation of polymer films using casting procedure: 1, plastic box; 2, thermostate; 3, thermostated metal surface; 4, thermogygrometer; 5, thermohygrodetector; 6, pump; 7, silica gel dryer; 8, thermoregulator; 9, cooler; 10, silica gel packs; a, silane pretreated upper glass support; b, photosensitive mixture of bacteriorhodopsin, gelatin, and additives; c, spacer; d, glass support. A chemically modified photochromic mixture of bacteriorhodopsin derivatives (14-F bacteriorhodopsin, both WT and D97N mutant), water-soluble polymer (gelatin), and chemical additives was introduced into the space between two glass supports separated by 1010-µm spacers and held at 9°C. The upper glass support was pretreated with dimethyldichlorosilane to produce a nonstick surface. After 1 h, the upper plate was removed. The samples were dried at 9°C and 13–15% RH.

during the bleaching procedure. After 1 to 2 d, purple membranes were bleached to form the apomembranes with the formation of retinal oxime. The apomembranes were purified by centrifugation, and then the purified apomembranes were reconstituted with retinal analogs. Reconstitution was carried out under a dim red light at 25°C, pH 6.0, by incubating the pure apomembranes with fluororetinae analog solution in a proportion of 1:1.5 mol, respectively. The progress of the reconstitution of 14-F retinal analogs with the apomembranes made from WT bacteriorhodopsin and D96N bacteriorhodopsin was monitored spectrophotometrically at 570 nm.

Photochromic polymer (gelatin) films were prepared using a casting procedure, in which the photosensitive mixture of the bacteriorhodopsin derivatives (14-F bacteriorhodopsin, both WT and D96N mutant) and gelatin binder was introduced between two glass supports separated by 1010-µm spacers and allowed to gel at 9°C (Fig. 2). After 1 h, the upper glass support, pretreated with dimethyldichlorosilane, was removed and samples were allowed to dry at 9°C and 12 to 15% RH (Fig. 2). The process of film preparation has been previously described in detail *(14)*. The thickness of the

dried samples was approx 70 μm. Chemical enhancement of the films containing additives increased the lifetime of the M-state.

In the course of the experiments, sample cuts ($15 \times 10 \times 1$ mm^3) from the films were mounted in sealed cuvets. The desired RH values (9, 18, 29, 38, 53, 62, 75, 84, and 92% RH) were produced by equilibration of the films for at least 24 h in the vapors of saturated salt solutions at the bottom of these sealed cuvets.

All ultraviolet-visible (UV-VIS) absorption spectral data and photoinduced absorption changes (suspensions as well as films) were measured with a Diode-Array UV/VIS Spectrophotometer HP 8452A and a Uvikon 923. The maximum power density of the excitation light (Kodak 300-W, 120-V bulb Ektagraphic projector equipped with a yellow long-pass filter $\lambda > 500$ nm) was approx 30 mW/cm^2. For the measurements all the samples were enclosed at the sealed cuvets.

The light-on kinetic changes of absorbance were analyzed using a one-exponential model, while light-off kinetic changes were analyzed in terms of a sum of exponentials. It is well known that in the first portion of the photocycle, one exponential fit for the kinetic processes under light is quite justified. This function fits the experimental curves with sufficiently small residuals of 1 to 2%. For the second portion of the photocycle, when the protein donates a proton to the periplasm and the Schiff base takes the proton to the cytoplasm, more than one exponential fit is required. The sum of two or three exponentials fits the light-off kinetics with residuals of about 1 to 3% of the measured value.

Results

Data from the literature show that dehydration of dried purple membrane, in both a form of air-dried films or polymer films, caused the changes in the initial absorbance maxima and corresponding amplitudes *(24–26)*. Results similar to those found in the literature were observed as changes in the initial absorbances in 14-F bacteriorhodopsin gelatin films, both WT and D96N, at different RHs (Fig. 3). Blue shifts in the initial absorbance maxima along with significantly diminished absorption amplitudes were observed with decreasing RH. These findings are similar to the data previously reported for WT bacteriorhodopsin with native chromophore *(26)*.

Figures 4 and 5 present the results of illuminating the 14-F WT and 14-F D96N gelatin films at different RHs. Differential absorbance spectra "light minus dark" measured after illumination of the samples with no chemical additives demonstrate a slight rise in absorbance in the red region (around 660 nm) for 14-F WT gelatin films at RH between 84 and 92% (Fig. 4A). For 14-F D96N gelatin films, a rise in absorbance at the 600-nm range was not observed even at 92% (Fig. 4B).

Differential absorbance spectra measured after illumination of the chemically enhanced 14-F WT films (films with the chemical additives DAP and GuHCl) (Fig. 5) demonstrate the rise in absorbance at the red region,

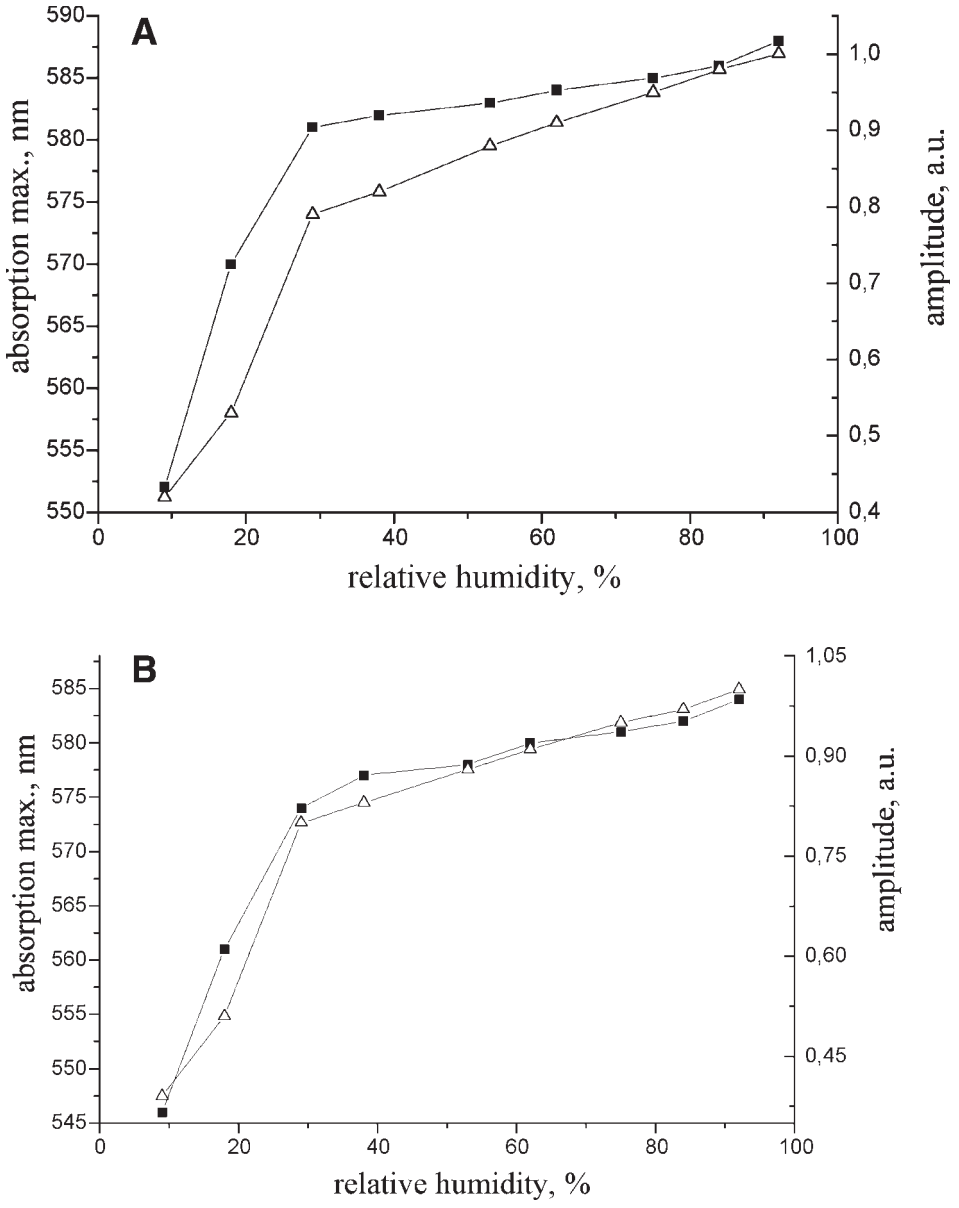

Fig. 3. Dependence of initial absorbance maxima (■) and relative amplitudes (△) on RH in chemically unmodified 14-F WT **(A)** and 14-F D96N **(B)** gelatin films. Amplitudes normalized to 1 arbitrary unit at 588 nm for 14-F WT and at 584 nm for 14-F D96N.

which is more pronounced at similar RH values when compared with the films with no additives. For 14-F D96N gelatin films, the addition of chemical additives did not result in any changes in photoinduced behavior in the red region as compared to films with no chemical additives at the same RH values.

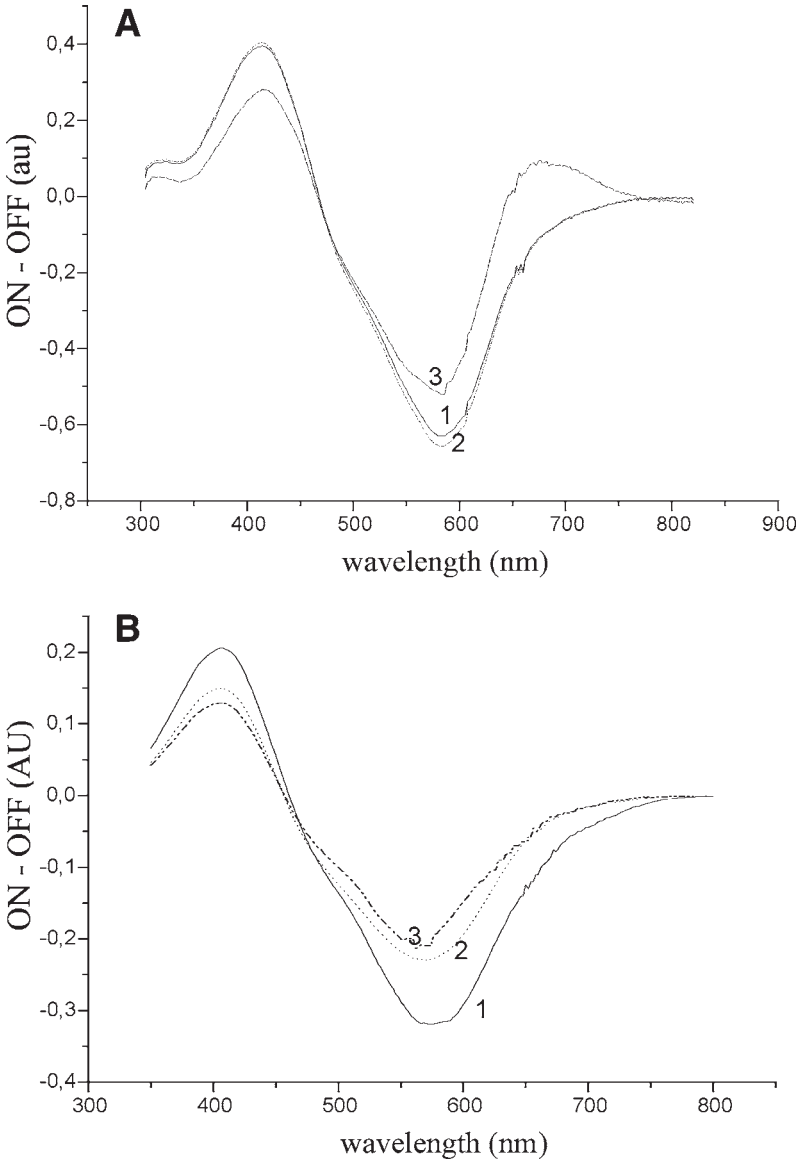

Fig. 4. Differential absorbance spectra (light minus dark) of **(A)** 14-F WT and **(B)** 14-F D96N gelatin films (no chemical additives) monitored at different RH values: solid curves 1 – at 75% RH, dashed curves 2 – at 84% RH, and dashed-dotted curves 3 – at 92% RH.

Kinetic measurements of photoinduced absorbance changes in 14-F WT and 14-F D96N gelatin films were monitored at 412, 588, and 660 nm and at 92% RH. For analysis of kinetic behavior, the time constants and corresponding relative amplitudes of the light-on and light-off processes in these films are presented in Table 1.

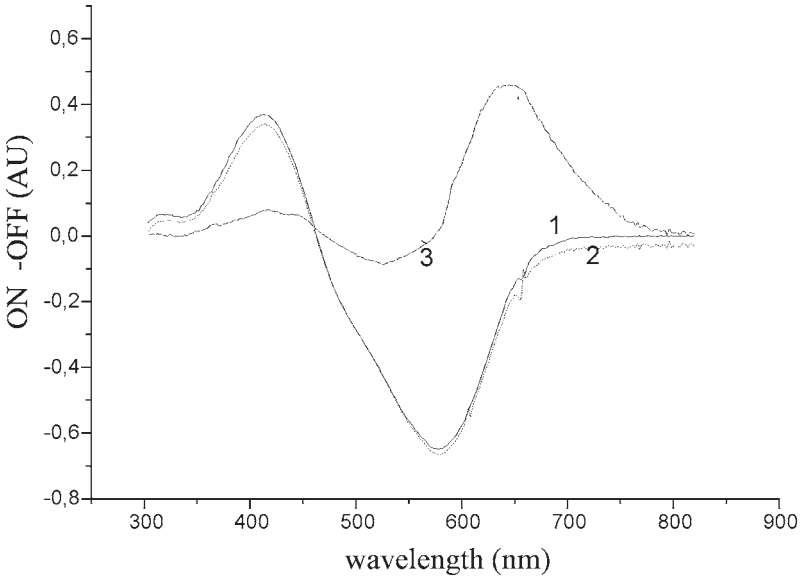

Fig. 5. Differential absorbance spectra (light minus dark) of 14-F WT gelatin films with chemical additives (DAP plus GuHCl) monitored at different RH of samples: solid curve 1 – at 75% RH, dashed curve 2 – at 84% RH, and dashed-dotted curve 3 – at 92% RH.

Discussion

Dramatic changes in the observed amplitudes at about 29% RH or less (Fig. 3) may be owing to dissociation of water molecules in the vicinity of retinal chromophore. This may induce deprotonation of the Shiff base *(27)*. The less rigid structure of the bacteriorhodopsin pigments with artificial chromophores may be the cause of the dramatic changes observed at RH much higher than for WT bacteriorhodopsin (between 12 and 3%) *(26)*. Study of native purple membrane by atomic force microscopy *(28)* shows that the loss of the Schiff base bond during photobleaching with hydroxyl amine (GA) leads to structural changes in the apoprotein. The process of photobleaching is associated with the disassembly of the purple membrane crystal into smaller crystals *(28)*. Although the regeneration of the photobleached membrane into a fully active purple membrane resulted in the reassembly of native structure, a small flexibility can be observed.

Thus, photoinduced changes in absorbance measured at <84% RH demonstrate no observed photoinduced formation of red-shifted species at 660 nm for either 14-F WT or 14-F D96N gelatin films. This is the opposite of what is observed for the corresponding pigments in water suspension *(21)*. In the range of 84–92% RH, red-shifted species with absorption at 660 nm were observed in 14-F WT gelatin films, but not in 14-F D96N gelatin films. Moreover, the chemical additives caused a significant accumulation of red-shifted species in these chemically enhanced 14-F WT gela-

Table 1
Time Constants of One-, Two-, or Three-Exponential Fits of Kinetic Curves Monitored at 412, 588, and 660 nm Under Light ($\lambda > 500$ nm) and After Light Exposure[a]

	14-F WT, without additives (τ_1, τ_2, $\tau_3 \pm$ SD) (s)	14-F WT, with additives (τ_1, τ_2, $\tau_3 \pm$ SD) (s)	14-F D96N, without additives (τ_1, τ_2, $\tau_3 \pm$ SD) (s)	14-F D96N, with additives (τ_1, τ_2, $\tau_3 \pm$ SD) (s)
412 nm light on	3.7 ± 0.41	3.01 ± 0.22	0.19 ± 0.067	0.38 ± 0.08
588 nm light on	2.47 ± 0.33	4.12 ± 0.66	0.68 ± 0.041	0.87 ± 0.074
660 nm light on	5.18 ± 0.67	4.48 ± 0.34	—	—
412 nm light off	0.99 ± 0.09	1.23 ± 0.43	8.67 ± 1.24	7.78 ± 1.13
	(0.28 ± 0.02)	(0.23 ± 0.03)	(0.39 ± 0.05)	(0.25 ± 0.04)
	10.67 ± 1.17	21.5 ± 1.45	17.32 ± 2.03	19.34 ± 2.07
	(0.63 ± 0.1)	(0.61 ± 0.09)	(0.23 ± 0.05)	(0.25 ± 0.05)
	318.4 ± 23.5	389.9 ± 34.5	263.5 ± 21.4	299.4 ± 34.1
	(0.08 ± 0.01)	(0.16 ± 0.1)	(0.38 ± 0.07)	(0.49 ± 0.01)
588 nm light off	1.66 ± 0.23	2.78 ± 0.34	6.45 ± 0.93	5.89 ± 0.74
	(0.19 ± 0.02)	(0.25 ± 0.04)	(0.43 ± 0.04)	(0.45 ± 0.08)
	18.55 ± 2.04	33.45 ± 6.9	12.03 ± 2.45	15.06 ± 2.56
	(0.65 ± 0.08)	(0.64 ± 0.08)	(0.27 ± 0.04)	(0.23 ± 0.03)
	401.5 ± 45.5	467.2 ± 56.7	256.2 ± 30.1	218.34 ± 39.1
	(0.15 ± 0.02)	(0.11 ± 0.01)	(0.28 ± 0.02)	(0.22 ± 0.04)
660 nm light off	57.34 ± 0.75	89.12 ± 10.2	6.15 ± 0.86	6.19 ± 0.71
	(0.58 ± 0.07)	(0.35 ± 0.05)	(0.58 ± 0.02)	(0.51 ± 0.05)
	253.12 ± 56.2	290.2 ± 35.8	178.3 ± 29.4	154.1 ± 29.4
	(0.41 ± 0.043)	(0.64 ± 0.01)	(0.40 ± 0.02)	(0.49 ± 0.05)

[a]The light-on kinetic changes in absorbance were analyzed using a one-exponential model. This function fits experimental curves with sufficiently small residuals of 1–3%. For the second portion of the photocycle, when the protein donates a proton to the periplasm and the Schiff base takes the proton to the cytoplasm, more than one exponential fit is required *(29)*. The sum of two or three exponentials fits the light-off kinetics with residuals of about 1–3% of the measured value. The SD is from three experiments. The relative amplitudes are given in parentheses under the corresponding time constants τ_i. The experiments were performed at room temperature (22 ± 1°C) and 92% RH.

tin films. It is likely that red-shifted species would also have been observed in 14-F D96N gelatin films if experiments could have been carried out at >92% RH, but this was not possible owing to technologic difficulties in handling totally wet films. Furthermore, samples at >95% RH cannot be strictly considered as dried films. The observed differences between WT and D96N mutant gelatin films in the formation of red-shifted species may result from the different kinetic characteristics of both pigments over the 84–92% RH range. When the kinetic characteristics of 14-F WT gelatin films at 92% RH (Table 1) were compared with those at 43% RH *(22)* and those of 14-F WT in water suspension *(21)*, it became apparent that they are close to those of the latter. By contrast, comparison of kinetic characteristics of 14-F D96N gelatin films at 92% RH (Table 1) with those at 43% RH *(22)* and 14-F D96N in water suspension *(21)* showed similarities between the results

obtained at 92 and 43% RH. We suggest the following explanation for the differences between the 14-F WT and 14-F D96N gelatin films. There was already evidence for the existence of two processes initiated in both 14-F WT and 14-F D96N by yellow light *(21)*. One of the processes is a photoinduced transformation of the initial state at 588 nm. The other is the formation and decay of red-shifted species at 660 nm. Therefore, the relationship between the rates of these two processes may be the reason for the observed differences in photoinduced behavior between the two types of gelatin films.

The differences in photoinduced behavior between these two types of films suggest that the hydration state of 14-F WT films in the range of 84 to 92% RH permits the observation of both processes similarly to that observed for 14-F WT in water suspension. The fact that chemical additives cause a significant accumulation of red-shifted species in these chemically enhanced 14-F WT gelatin films at this RH range confirms that at these conditions the photoinduced processes in gelatin films parallel those of 14-F WT in water suspension at pH 7.0–9.0 *(21)*. In the case of 14-F D96N gelatin films, it is suggested that the vicinity of the chromophore in the range of 84 to 92% RH is still the same as at an RH <84%. This is confirmed by the fact that the maximum of initial absorbance at 92% RH is 584 nm, as compared with 588 nm for the water suspension *(21)*.

In conclusion, 14F-WT gelatin films demonstrate the desired properties (formation of red-shifted species) in the range of 84 to 92% RH and may offer technological advantages relative to those based on 14-F D96N. Gelatin films such as those based on 14-F WT can be used in various devices of optical memory in which recording and processing of optical information may carried out using semiconductor lasers as the light source.

Acknowledgment

This research was supported in part by grant no. 01-04-48198 from the Russian Foundation of Basic Research.

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