

# The photocycle of bacteriorhodopsin immobilized in poly(vinyl alcohol) film

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The kinetics of photoelectric and optical signals were measured on samples containing oriented purple membranes immobilized in a poly(vinyl alcohol) film and on purple membranes introduced into a PVA–H<sub>2</sub>O mixture. The bacteriorhodopsin photocycle in the PVA–H<sub>2</sub>O mixture was complete. The only observed changes were the slowing down of the optical and electrical signals in relation to the M<sub>412</sub>–O<sub>640</sub> and O<sub>640</sub>–bR<sup>sl 1-trans</sup> steps. In the PVA film the O<sub>640</sub> intermediate disappeared and a negative photoelectric signal appeared.

Bacteriorhodopsin; Photocycle; Photoelectric signal; Oriented system

## 1. INTRODUCTION

Bacteriorhodopsin is the only protein of the purple membranes of *Halobacterium halobium*. On absorbing photons bR runs through photocycles and the protons are translocated across the membrane. Mainly 4 intermediates: K<sub>590</sub>, L<sub>550</sub>, M<sub>412</sub> and O<sub>640</sub> with lifetimes in the micro- and millisecond range have been well characterized [1]. The fast flash-induced electric and absorption signals from bR have been measured on several model systems: on purple membranes attached to planar lipid membranes [2], on pm immobilized in gel [3] and on dry oriented pm samples [4]. The choice of the proper model system depends on the phenomenon of bR that is intended to be studied.

There are two current models for long-range transport of protons through bR molecules. The so-called 'charge injection model' assumes the existence of hydrogen-bonded chains built up from special amino acid side chains of bR [5]. The other model does not require any special structure as it is based on the general properties of proteins. It is assumed that protons in the M<sub>412</sub> state are taken over by H<sub>2</sub>O molecules in the protein and the H<sub>3</sub>O<sup>+</sup> ions are driven out to the external side by the existing internal electric field due to the primary charge separation [6].

The latter model was partially supported by substantial changes of the Arrhenius parameters and the

necessity of fluid bulk water for the appearance of the O<sub>640</sub> state which is essential for proton pumping [7]. However, there is still a lack of crucial arguments definitely convincing, which proton pumping mechanism is correct. Therefore it should be necessary to find a proper model system in which the parameters of the purple membranes environment (unequivocally characterizing the proton-pumping mechanism) could be changed. Such a model system would be able to mimic conditions necessary for proper bR activity.

In our previous study [8] we stated that bacteriorhodopsin behaved differently in liquid PVA and in PVA film. These studies conveniently demonstrate the influence of free water on the bR photocycle and hence might be useful in emphasizing the correctness of the latter proton pumping model. Therefore, detailed studies of the protein electric response signals and absorbance changes of the pm–PVA liquid solutions or films are presented here.

## 2. MATERIALS AND METHODS

Purple membrane fragments were isolated from *Halobacterium halobium* strain ET 1001 by the standard procedure [9]. The pm suspension was mixed with 13% aqueous solution of PVA (Fluka AG, without further purification).

Sample preparation was performed according to Varo [10]. The absorbance of the samples at  $\lambda = 570$  nm was slightly smaller than 0.8 OD for optical measurements and about 2 OD for photoelectrical measurements.

The measurements set-up of the photoelectric and optical signals reported earlier [11] was extended. The sample was placed on a horizontal table. The photomultiplier was positioned above the sample. Owing to the horizontal position of the glass slide, the measurements could have been performed during sample drying (Fig. 1).

**Abbreviations:** bR, bacteriorhodopsin; pm, purple membrane; PVA, poly(vinyl alcohol); PERS, protein electric response signal.

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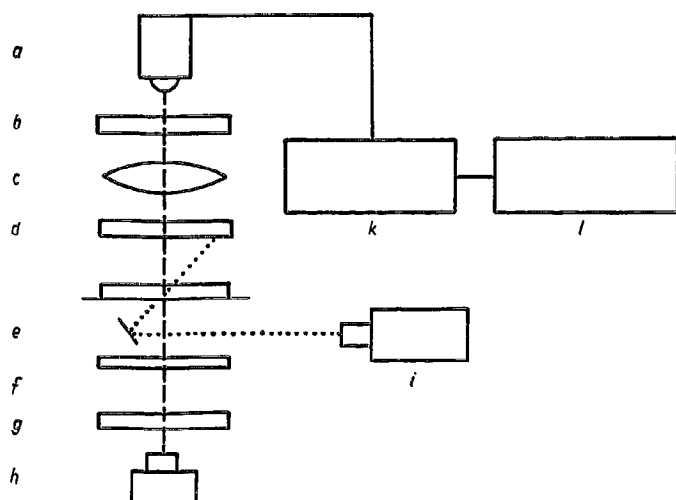


Fig. 1. Scheme of the measuring set-up. a, photomultiplier; b, interference filter; c, lens; d, polarizer; e, mirror; f, heat filter; g, lamp; h, laser; i, amplifier; k, transient recorder; l, multichannel analyzer.

The thermogravimetric measurements were made using a Mettler Thermoanalyzer. The rate of heating ranged from 0.5 to 4°C/min. There were no differences between thermogravimetric traces obtained at different heating rates (the results are not shown).

### 3. RESULTS AND DISCUSSION

The obtained typical electric signals of the purple membranes and changes of the intensity of light passing through the samples (these changes will be further called 'optical signals') are shown in Fig. 2. Optical signals at different wavelengths were measured:  $\lambda_1 = 525$  nm,  $\lambda_2 = 403$  nm and  $\lambda_3 = 635$  nm. These signals were related to the absorbance changes caused by  $bR^{a11-trans}$ ,  $M_{412}$  and  $O_{640}$  intermediates respectively. Both electric and

optical signals were fitted with different numbers of exponentials. The time constants of these signals are summarized in Table I and Table II.

After incorporation of pm in the aqueous PVA solution all intermediates of the bR photocycle were observed. The kinetics of the  $L_{550}$ - $M_{412}$  transition were almost identical as for bR of pm in water while the time of the  $M_{412}$ - $O_{640}$  and  $O_{640}$ - $bR^{a11-trans}$  transitions was slowed down by two to three orders of magnitude (compare Table I and [1,10]).

The slow part of the bacteriorhodopsin photocycle is very sensitive to change of the physico-chemical conditions of the purple membrane environment [11]. Thus an increase of the pm environment viscosity (water-PVA solution in comparison with water) can slow down this part of the bR photocycle. A similar phenomenon was observed by Beece et al. [12] for pm incorporated into a water-glycerine mixture. It seems possible that bR can contact free water molecules in a very viscous solvent (as the water-glycerine mixture or liquid PVA). But this contact is more difficult than when the pm environment contains only water. Because of this, signals exist that can be related to  $bR^{a11-trans}$ ,  $M_{412}$  and  $O_{640}$  forms for pm in liquid PVA but the slow part of the bR photocycle was retarded.

As water evaporates from the sample the photocycle of bR is changed. This can be observed on the basis of the photoelectric and optical signals corresponding to the proper parts of this photocycle. The most striking difference is the slow negative PERS corresponding to the  $M_{412}$ - $bR^{a11-trans}$  transition and the disappearance of the optical signal related to the  $O_{640}$  form. This negative signal has never been measured in the pm suspension [13]. Nevertheless, a similar negative PERS was observed for dried oriented samples by Varo and Keszthely

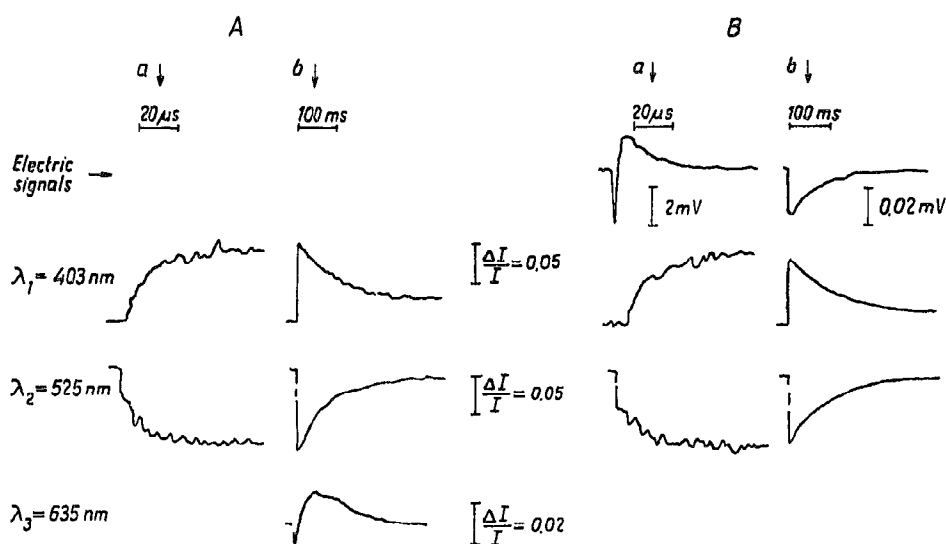


Fig. 2. Comparison of electric and optical signals obtained from a pm-PVA liquid suspension (A) and pm-PVA film (B). a and b, signals measured with different time resolution.  $I$ , current caused by light passing through sample;  $\Delta I/I$ , change of current ( $I$ ) caused by proper absorbance changes.

Table I  
Time constants of the optical signals

| Signal type<br>$\lambda$ [nm] | pm susp. in<br>PVA-H <sub>2</sub> O |              | pm in PVA film     |          |              |          |
|-------------------------------|-------------------------------------|--------------|--------------------|----------|--------------|----------|
|                               | components                          |              | components         |          |              |          |
|                               | fast<br>( $\mu$ s)                  | (slow<br>ms) | fast<br>( $\mu$ s) |          | slow<br>(ms) |          |
|                               | $\tau$                              | $\tau$       | $\tau_1$           | $\tau_2$ | $\tau_1$     | $\tau_2$ |
| 525                           | 59                                  | 920          | 25                 | 65       | 37           | 970      |
| 403                           | 58                                  | 921          | 12                 | 61       | 41           | 963      |
| 635                           | 97 ms                               | 827 ms       | -                  | -        | -            | -        |

Table II  
Time constants of PERS

| Sample No. | pm in PVA film  |          |           |          |
|------------|-----------------|----------|-----------|----------|
|            | components      |          |           |          |
|            | fast ( $\mu$ s) |          | slow (ms) |          |
|            | $\tau_1$        | $\tau_2$ | $\tau_1$  | $\tau_2$ |
| 1          | 18              | 56       | 29        | 1087     |
| 5          | 25              | 48       | 42        | 985      |
| 9          | 23              | 51       | 32        | 1024     |

[11]. They suggested that this signal had been caused by the backflow of proton in bR. The similarity also concerns the character of the signal's decay (or rise). In the case of pm incorporated into a PVA film both electric and optical signals were better fitted with two exponentials (Tables I and II). Because of the difficulty in stabilizing the electric resistances and capacitance of the pm-PVA mixture during its drying, PERS of the pm-PVA film was only measured.

Owing to the horizontal position of the glass slide (Fig. 1) the changes of the absorbance related to the O<sub>640</sub> form were measured during sample drying. These signals are presented in Fig. 3. During the PVA-pm

film creation, the character of the generated absorbance changes was the same. This shows that the time constants of these optical signals remained almost unchanged and were similar to those obtained for the pm-PVA liquid solution (compare Table I and data from Fig. 3).

As seen in Fig. 3 the shape of the observed signals was not influenced by sample drying but the amplitudes of these signals decreased. This decrease was not linear with the time of sample drying. It is possible to distinguish 3 regions of drying (Fig. 4). The first region: about 6 h of sample drying after formation of the pm-PVA liquid suspension. A small, almost insignificant decrease of the optical signal amplitude was observed during this period. The second region: between the 6th and 10th hour of drying. A drastic decrease of the optical signal amplitude was registered. The third, last region: between the 10th and 15th hour of drying. No optical signal related to the O<sub>640</sub> form was observed. These results suggest that if the amount of water drops below a somehow defined level for bR, the optical signal connected with the O<sub>640</sub> form disappears.

The PVA film kept at room temperature contains approximately 25–30% of water that is adsorbed on the film surface and is involved in the PVA structure [14].

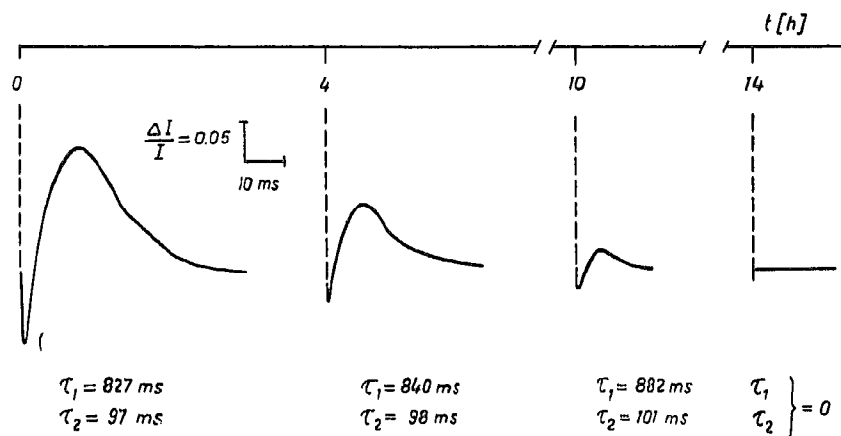


Fig. 3. Changes of optical signals related to O<sub>640</sub> form during sample drying (measured in hours;  $\tau_1$  and  $\tau_2$ , decay times).

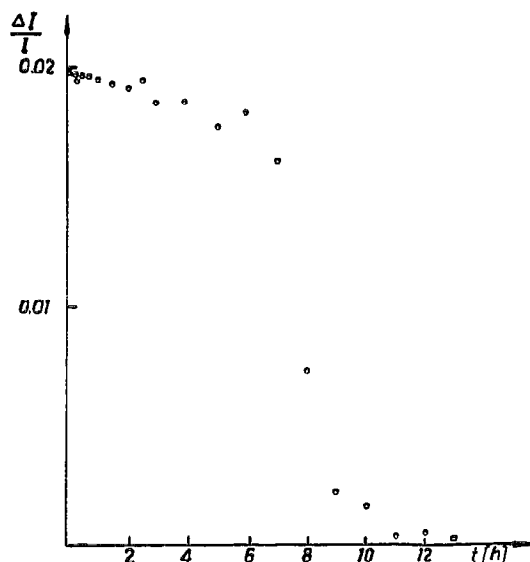


Fig. 4. Changes of optical signals amplitudes related to the  $O_{640}$  form during sample drying.

Warming of the PVA film up to  $50^{\circ}\text{C}$  does not change the water content of the sample (Fig. 5). Between  $50^{\circ}\text{C}$  and  $60^{\circ}\text{C}$  a small fraction of total water included in the PVA film is removed. The temperature range of this process indicates with great probability that the adsorbed water is at this moment released [15]. But, as seen in Fig. 5, only prolonged warming of the PVA film at a temperature of  $120^{\circ}\text{C}$  can afford complete removal of the remaining water molecules from the PVA film. This shows that this water is not free (in the sense of water categories existing in biological systems [16]). The PVA-pm films contain more water in comparison with PVA films because some of the water molecules are carried with purple membranes (the upper curve in Fig. 5). The shape of the thermogravimetric curve indicates that the same category of water molecules exists in this

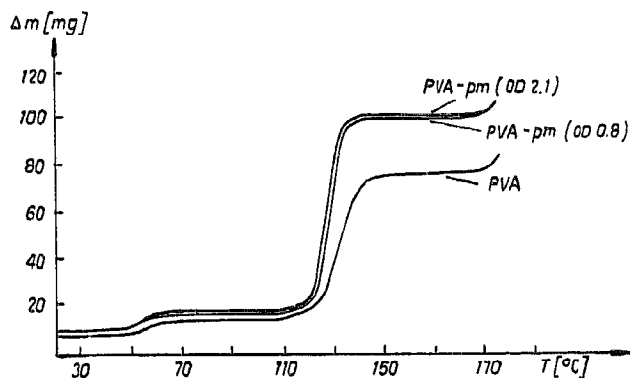


Fig. 5. Thermogravimetric analysis of the water content of the PVA and pm-PVA samples. ( $\Delta m$ , change of the sample mass during warming it up, % change of the water content; samples excluding masses were approx. 373 mg).

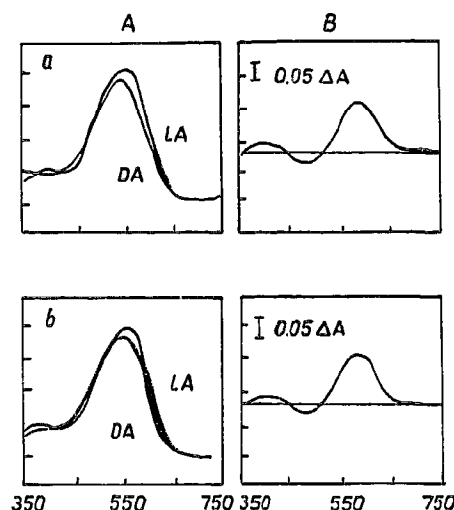


Fig. 6. (A) Absorption spectra of pm-PVA liquid suspension (a) and pm-PVA film (b). DA, dark adapted bR; LA, light adapted bR. (B) Difference spectra.

system. Thus the energy needed to remove water molecules from the pm-PVA system is of the same order of magnitude as the energy for the PVA film. The suggestion that free water is initially removed from the samples is further supported in Fig. 6, in which the absorption spectra of light- and dark-adapted bR are presented. There is no difference between the absorption spectra showing a decrease of the number of water molecules in the interior of bR. Thus the process of water removal from the samples does not interfere with the water inside the bR structure. Therefore the amount of bound water can be unchanged.

The results presented in this work suggest that there is mostly bound water in the pm-PVA film at room temperature but this water cannot play the role of proton carrier. A very small fraction of total water in the pm-PVA film that can be treated as free water is not sufficient for preserving the complete bR photocycle. Hence, the step in which the free  $\text{H}_2\text{O}$  molecule binds a proton and moves as an  $\text{H}_3\text{O}^+$  ion disappears. Thus there is no step leading to the  $O_{640}$  form. The bR photocycle is shortened and instead of a proton movement outside the purple membrane the proton goes back.

Such a mechanism of proton movement in a pm-PVA film system is in accordance with the model of bR proton pumping that was originally proposed by Keszthelyi et al. [6]. Therefore the results presented above support this model.

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