THE PHOTOCYCLE OF BACTERIORHODOPSIN IN THE TWO-DIMENSIONAL ORTHOROMBIC CRYSTAL FORM OF PURPLE MEMBRANE

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ABSTRACT Laser flash photolysis and low-temperature absorption studies of the photocycle of orthorhombic purple membrane (o-PM) reveal the existence of the same K, L, and M intermediates as found in the native hexagonal purple membrane (h-PM). However, the 0 intermediate is missing in the o-PM. The absorption spectrum of the K intermediate of o-PM is blueshifted by ~15 nm relative to the K intermediate found in the hexagonal purple membrane. The decay relaxation time constants of M in the o-PM are higher by more than an order of magnitude than the corresponding relaxation time constants in the h-PM. Similarly to the h-PM, the decay of M depends on the pulse width of excitation. The time-independent anisotropy factor obtained in photoselection studies of the M intermediate demonstrates the complete immobility of bacteriorhodopsin (bR) within the o-PM matrix. The same anisotropy factor of 0.3 obtained for o-PM and for h-PM suggests that in both crystalline lattices the transition moment of the retinal chromophore has similar angles with the plane of the membrane. The dependence of the decay kinetics of M on its occupancy may suggest the existence of kinetic coupling between neighboring bR molecules.

INTRODUCTION

Bacteriorhodopsin (bR), the only protein in the purple membrane, undergoes a photochemical cycle upon illumination (for review see references 1, 2, 3). During this photocycle, protons are released on one side of the membrane. Protons are taken up from the other side of the membrane during restoration of the original state, so that an electrochemical proton gradient can be built up across the membrane.

In the native purple membrane, trimers of bR form a hexagonal two-dimensional lattice of space group[1] p3 (5). The arrangement of bR molecules imposes a strong protein-protein interaction within the trimers, and probably also between different trimers. However, it cannot be excluded that interactions between different trimers occur solely indirectly via bound lipid. It has been suggested that a kinetic coupling exists between the bR molecules in the trimers (6, 7).

Recently, a new two-dimensional crystal form of purple membrane has been obtained in vitro (8, 9). This new form of purple membrane is orthorhombic with space group $p22_12_1$. There are four bR molecules in the unit cell, and the areas per bR molecule are similar in both crystal forms. The bR molecules have identical molecular structure, but the protein-protein interactions are different in the two crystal forms. It is therefore of interest to note how different protein-protein interactions can affect photocycle and optical properties. Here we compare the bR photocycle and other optical properties in the hexagonal and orthorhombic crystal forms of purple membrane.

MATERIALS AND METHODS

Hexagonal purple membrane (h-PM) was isolated from *Halobacterium halobium* NRL R_1M_1 (10). Orthorhombic purple membrane (o-PM) was prepared according to method A in references 8 and 9. o-PM was suspended in D_2O (99.9% Carl Roth, Karlsruhe, Federal Republic of Germany) as previously described (11).

The kinetics, as well as the absorption difference spectra, were measured by the experimental apparatus shown in Fig. 1. The sample was excited by a 400-W quarz-iodine lamp (LS_1). Its light was filtered by a neutral density filter (F_2) and a cut-off Eppendorf filter >560 nm (F_1). Alternatively the light from a flash lamp-pumped rhodamine 6G dye laser (Carl Zeiss, Oberkochen, Federal Republic of Germany) was used for excitation. In this case the photocycle kinetics were determined by single-flash excitation. The measuring light from a 400-W quarz-iodine lamp (LS_2) was passed through a Zeiss monochromator (M) (M4QIII; Carl Zeiss, Inc., Thorwood, NY), was focused onto the cuvette and was then passed through another Zeiss monochromator (M) (Carl Zeiss, Inc.) to the photomultiplier (PM) (EMI 9634 QR; EMI, Inc., Clinton, CT). The output of the photomultiplier was amplified and passed through a variable electrical RC filter to a digital oscilloscope (Nicolet 1090A Explorer, model 96A; Nicolet Instrument Corp., Madison, WI). The data were transferred to a computer.

For transient dichroic measurement, thin o-PM layers attached to glass slides were prepared as shown previously (12). The photoselection measurements were carried out by irradiating the thin purple-membrane layer with light >560 nm (LS_1) through a Glan-Taylor ultraviolet prism polarizer (Spindler and Hoyer GmbH and Co., Göttingen, Federal

[1]We use Holser's nomenclature (4) for two-dimensional space groups.

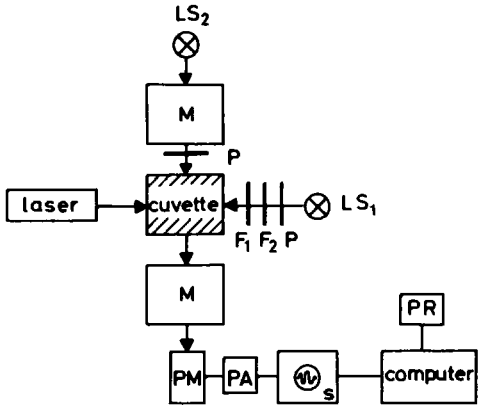

FIGURE 1 A block diagram of the experimental set-up is illustrated: F_1, cut-off Eppendorf filter; F_2, neutral filters; P, polarizer; LS_1, LS_2, lamps; M, monochromator; PM, photomultiplier; PA, preamplifier; S, oscilloscope; PR, printer.

Republic of Germany). The photoselection was performed far from saturating light conditions. Both excitation and analysis light axes formed simultaneously an angle of 45° with the plane of the glass slide. The anisotropy factor $r(t)$ measured in these photoselection experiments is defined as

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)},$$

where $A_{\parallel}$ and $A_{\perp}$ are absorption values at 412 nm with the analysis polarizer, parallel and perpendicular to the exitation polarized light, respectively.

The decay of M photocycle intermediate[2] was fitted by a sum of exponentials. The initial values for the rate constants and their relative amplitudes were calculated by a computer program, based on a nonlinear approximation (Müller, K. H., and T. Plesser, unpublished data). These results were optimized by a least-square program (Harwell Subroutine Library, VC05A, Computer Science and Systems Division, Aere Harwell, Oxfordshire, England). The relaxation time constants given are the inverse of the rate constants. Different photostationary concentrations of M were produced by >560 nm steady flux light from LS_1 lamp, where the intensity was modulated by various neutral filters (F_2), and the off reaction was analyzed as a sum of exponentials.

The pulse width of the excitation light from LS_1 lamp was determined by a mechanical shutter. Since the decay kinetics depend on the ratio of (M)/(bR) (6), the intensity of the longer light pulses was decreased by means of neutral filters so that the decay was measured from the same low concentration level of M.

Low-temperature spectra of photocycle intermediates in their photostationary states were measured using a Cary 14 spectrophotometer (Varian Associates, Instrument Div., Palo Alto, CA) equipped with a low temperature cooling system as described previously (14). Low-temperature absorption spectra of the photocycle intermediates were taken using thin o-PM and h-PM films. These films were prepared and then equilibrated with 94% relative humidity by a procedure similar to that used for h-PM (15, 16). The advantage of using hydrated films over glycerol-water solvent mixtures for low-temperature studies is that changes in light scattering do not occur in thin layers of purple membrane attached to the surface of a cuvette.

The extent of phototransformation of bR into its K photocycle intermediate at −180°C (Fig. 4) was calculated from two photostationary states obtained by irradiation at 546 and 578 nm with a low pressure 125-W mercury lamp. The calculation is based on an approximation introduced by Fischer (17) in the calculation of the extent of conversion for A ⇌ B system when only the absorption of A is known. This method allows a determination with an accuracy of ±10%. Both for h-PM and o-PM we assumed the quantum efficiencies at 546 and 578 nm to be similar. For the h-PM this assumption is correct because we can calculate from a study on the wavelength dependence of the quantum efficiency for h-PM (21) that efficiencies at 546 and 578 nm differ by <10%.

[2]The abbreviations for the photocycle intermediates are taken from those given in reference 13.

RESULTS AND DISCUSSION

Absorption Spectra of the Photocycle Intermediates

The photocycle of the o-PM was investigated both by laser flash photolysis in aqueous solutions at room temperature and by stationary methods in thin hydrated films at low temperatures. The dark-light adaption of h-PM, which is accompanied by 13-*cis* to all-*trans* photoisomerization of the retinal chromophore (18), was shown to depend on the hydration state (15). A complete conversion of a dark-adapted, fully hydrated (94% relative humidity) thin o-PM film into the light-adapted form is shown in Fig. 2. This dark-light adaptation, which is similar to that observed in aqueous suspensions of o-PM (9), demonstrates the identical *cis-trans* photoisomerization characteristics of o-PM under both conditions.

To obtain the absorption spectrum of the longest-lived M intermediate in o-PM, irradiation with light >500 nm was carried out at −40°C (Fig. 3, curves *1* and *2*). The M intermediate is not thermally stable at −40°C and slowly decays into the initial bR state (Fig. 3, curves *3–10*), but the decay is not a single exponential one. It could be fitted as a sum of two exponentials. Furthermore, irradiation of M at 405 nm yields nearly complete photoconversion of the M state into the initial bR state (~97%). To obtain the pure absorption spectrum of M, we have extrapolated curve *2* in Fig. 3 to 100% conversion, obtaining curve *11* in Fig. 3. The extrapolated absorption of M yields $\epsilon_M^{412} \sim \epsilon_{bR}^{564}$.

Irradiation of o-PM at temperatures lower than −60°C yields a mixture of M and L forms. The formation of M accompanies the formation of L intermediates down to −160°C. This result is different from that for h-PM, where the M intermediate is not formed at temperatures lower than −120°C.

Irradiation of o-PM and h-PM at −180°C yields photostationary state mixtures of bR and K (Fig. 4 *a,b*). The existence of the apparent isobestic point at 578 nm and 587 nm for the h-PM and o-PM, respectively, suggests that at this temperature a photoequilibrium, mainly between two forms, is established. To obtain the pure absorption spectrum of K, the percent of conversion into the K intermediate for a given photostationary state has to be determined. The procedure based on raising the temperature of the photostationary mixture of K and bR, which would lead to a full conversion of K into M, could not be used. Even under experimental condition where M is thermally stable full conversion into M was not obtained. This might

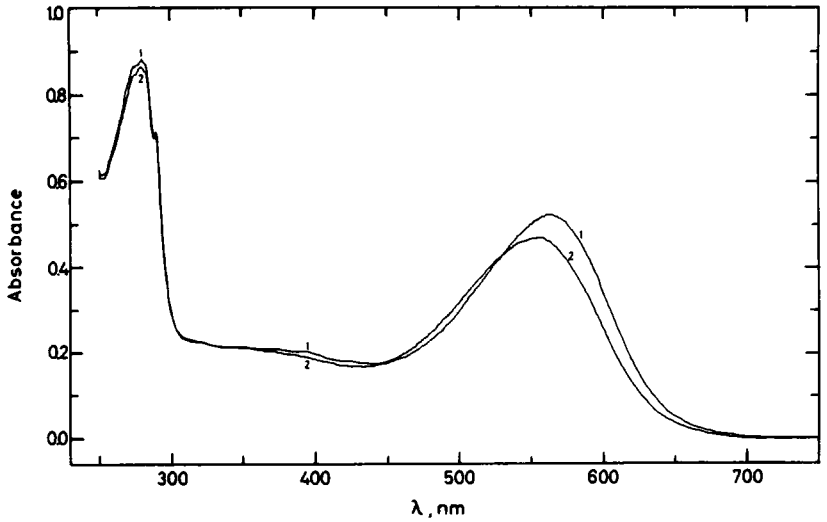

FIGURE 2 The absorption spectra of thin layers of o-PM equilibrated with 94% relative humidity of 22°C are shown: curve *1*, light-adapted orthorhombic purple membrane obtained from curve *2* by irradiation at 500 nm to a photostationary state; curve *2*, dark-adapted o-PM.

be due to the existence of a bypass in the decay of the L intermediate as was found for h-PM (19). Therefore, an approximation based on reference 17 was used to determine the amount of 20% K present in the photostationary state obtained by irradiation at 578 nm for both o-PM and h-PM. Extrapolation of the spectra in Fig. 4 *a,b* curve *2* to 100% conversion of K yields the pure absorption spectrum of K (Fig. 4, curve *4*). The spectrum of the K intermediate in o-PM obtained by this procedure shows a maximum at 605 nm, which is blueshifted relative to the spectrum of the K intermediate in hexagonal purple membrane (maximum at ~620 nm). Furthermore, a difference in the ratio of the extinction coefficients for K and bR in the two crystalline forms can be observed.

Photocycle Kinetics of the Orthorhombic Purple Membranes

The longest-lived intermediates in the photocycle of the o-PM and the h-PM were studied by laser flash photolysis. The absorption spectra obtained for M in the orthorhombic and hexagonal crystal forms are similar (Fig. 5 *a,b*). However, the existence of the 0 intermediate could not be detected in the orthorhombic form under single flash

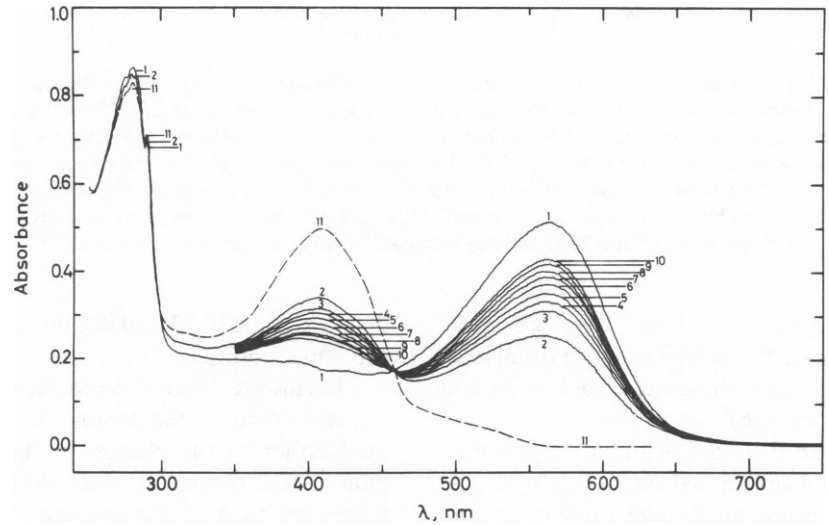

FIGURE 3 The absorption spectra of photostationary state of mixtures of o-PM and M in thin layers of o-PM equilibrated with 94% relative humidity, at −40°C are shown: curve *1*, before irradiation; curve *2*, after irradiation with 500 nm to a photostationary state. Curves *3–10* represent the thermal decay of M, the spectra represented by curves *3–10* were taken at different times after the irradiation as follows: curve *3*, 5.5 min; curve *4*, 11.5 min; curve *5*, 24 min; curve *6*, 39 min; curve *7*, 59 min; curve *8*, 90 min; curve *9*, 130 min; curve *10*, 190 min. Curve *11* gives the pure absorption spectrum of M obtained by extrapolation of curve 2 to 100% conversion into M.

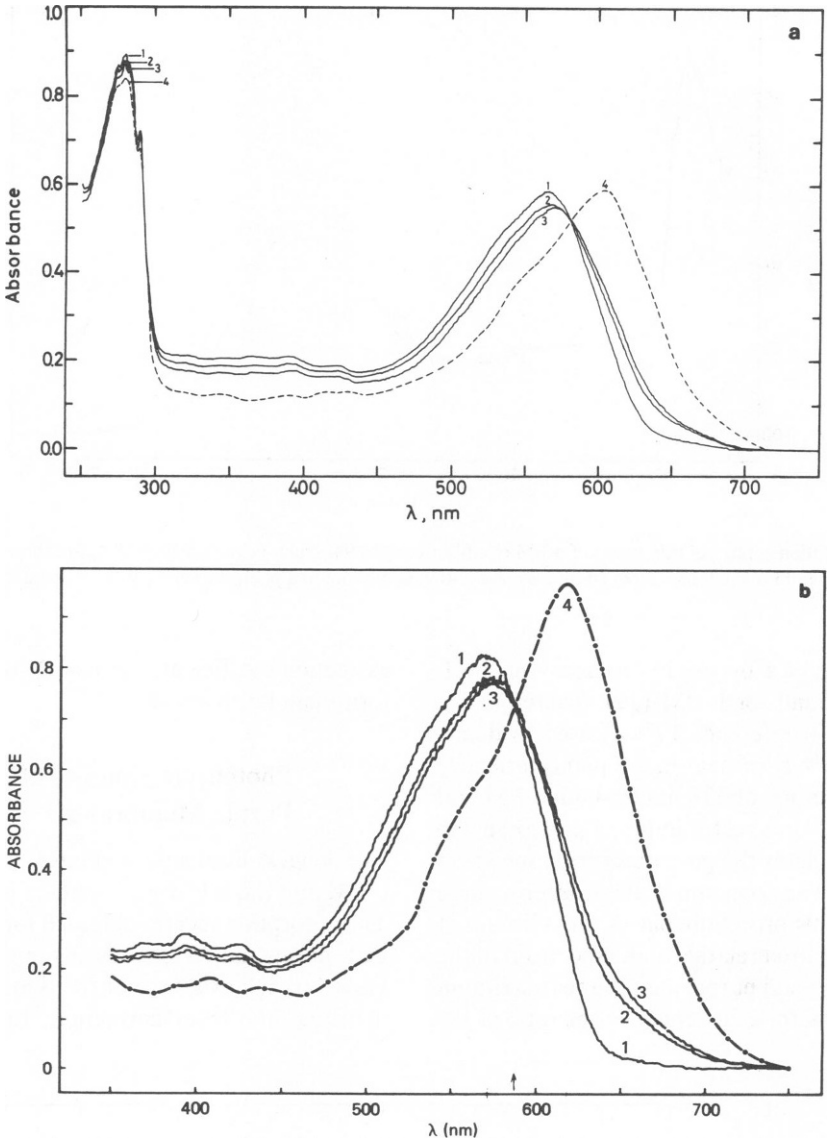

FIGURE 4 (*a*) The absorption spectra of photostationary state mixtures of o-PM and K in thin layers of o-PM equilibrated with 94% relative humidity, at −180°C are shown: curve *1*, before irradiation; curve *2*, photostationary-state mixture of o-PM and K produced by irradiation at 578 nm; curve *3*, photostationary-state mixture of o-PM and K produced by irradiation at 546 nm; curve *4*, the pure absorption spectrum of K obtained by extrapolation using the method in reference 17. (*b*) The absorption spectra of photostationary state mixtures of h-PM and K in thin layers of h-PM equilibrated with 94% relative humidity, at −180°C are shown. Curve *1*, before irradiation, curve *2*, photostationary state mixture of h-PM and K produced by irradiation at 578 nm; curve *3*, photostationary state mixture of h-PM and K produced by irradiation at 546 nm; curve *4*, the pure absorption spectrum of K obtained by extrapolation using the method in reference 17.

excitation at room temperature. It has been previously shown that the appearance of 0 is very sensitive to environmental perturbation, such as temperature, pH, or hydration state of the membrane (16).

The decay of M in the o-PM was not a single exponential one. It could be fitted as a sum of two exponentials yielding relaxation time constants and amplitudes (in parentheses) of τ_1 = 130.4 ± 2.7 ms (0.66 ± 0.1) and τ_2 = 1,203.7 ± 32.5 ms (0.32 ± 0.01) at 6°C (50 mM phosphate buffer, pH 7). These decay relaxation time constants are more than one order of magnitude higher than the relaxation time constants, τ_1 = 10.1 ± 1.0 ms (0.46 ± 0.09) and τ_2 = 26.6 ± 2.4 ms (0.52 ± 0.08) obtained for the h-PM under the same conditions.

The slowing down of the M decay and the absence of the 0 intermediate in the photocycle of o-PM may be attributed either to the change in the packing mode of bR molecules in the orthorhombic form or may arise from the procedure used in the preparation of o-PM, especially, since ^{3}H-Triton X-100 could never be removed completely. Always a substoichiometric amount of Triton (molar ratio of Triton/bacteriorhodopsin ≤0.3) was still found.

A control experiment performed with h-PM in the presence of Triton X-100 and cetyltrimethyl ammonium

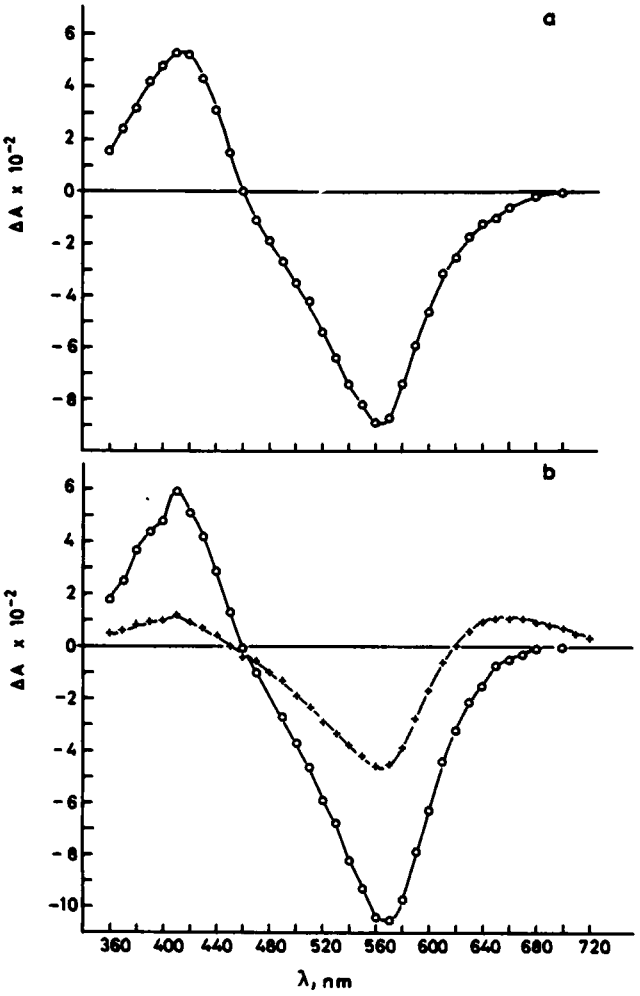

FIGURE 5 Transient absorption difference spectra produced by laser flash excitation of aqueous suspensions of h-PM and o-PM with 50 mM phosphate buffer pH 7.0 are shown (*a*) o-PM; maximum absorption change was taken 1 ms after excitation at 5°C. (*b*) h-PM; the absorption change was taken after 0.5 ms (o) and 5 ms (+) after excitation at 22°C.

bromide (CTAB) showed that even under conditions of large excess of detergent (e.g., molar ratio of Triton/CTAB/bR of 1:10:1 the first and second relaxation time constants of the M intermediate were slowed only by a factor of 2 and 3, respectively. This observation together with the fact that 0 intermediates could be observed under these conditions (though its amplitude was reduced by 55% in controlled experiment with detergents) suggests that the difference in the kinetic or photochemical properties of bR in the o-PM and h-PM probably do not arrive from the pretreatment with detergents during the precrystallization stage. It is therefore likely that the arrangement of bR in the native h-PM can accelerate the bR photocycle.

As was shown for h-PM (20) the decay of the M intermediate is dependent of the pulse width of excitation (Fig. 6, Table I). However, whereas the relaxation time constants obtained by laser and steady light excitation vary

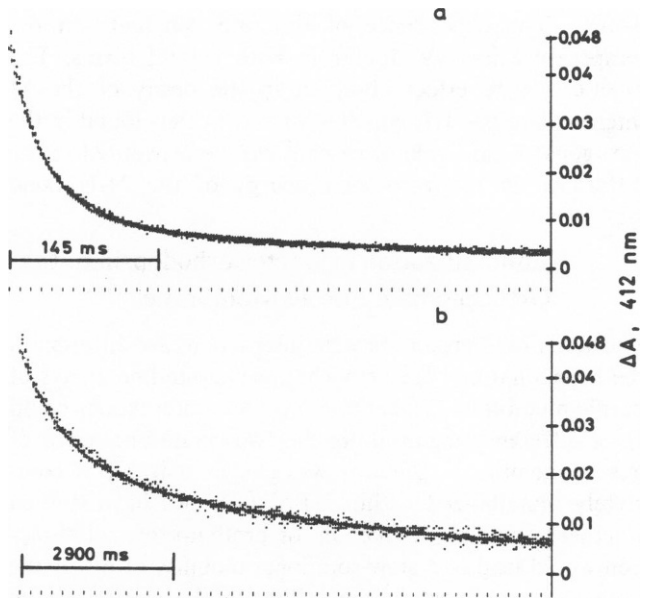

FIGURE 6 The decay kinetics of the M intermediate as function of the pulse width of light excitation in aqueous suspension of o-PM (50 mM phosphate buffer, pH 7.0) are shown: (*a*) decay kinetics of the M intermediates formed by a pulsed laser light (3 μs pulse width); (*b*) decay kinetics of the M intermediate formed by continuous light (infinite pulse width). Dots indicate the original data points, whereas crosses indicate the best fit data points when analyzed as a sum of two exponentials.

by a factor of 20 in the o-PM, the corresponding change in the hexagonal one is only fourfold. Such a dependence of the measured decay of the M intermediate on the preillumination time can be explained, if the M intermediate equilibrates with another M form (see also reference 20).

Rate constants for the decay of the M in H_2O and D_2O were measured over a wide temperature range of 2°C to

TABLE I
DECAY* OF M^{412} AS A FUNCTION OF PULSE WIDTH OF THE EXCITING LIGHT

Pulse width	τ_2	τ_1
	ms	*ms*
3 μs	283 ± 6	23 ± 1
	(0.20 ± 0.01)‡	(0.72 ± 0.01)
250 ms	1,083 ± 26	60 ± 3
	(0.036 ± 0.01)	(0.53 ± 0.01)
1 s	2,077 ± 116	173 ± 12
	(0.39 ± 0.01)	(0.44 ± 0.01)
2 s	2,307 ± 268	177 ± 11
	(0.41 ± 0.01)	(0.42 ± 0.01)
4 s	2,403 ± 46	191 ± 3
	(0.43 ± 0.01)	(0.40 ± 0.01)
8 s	3,690 ± 420	347 ± 72
	(0.34 ± 0.02)	(0.44 ± 0.01)
∞§	4,668 ± 450	431 ± 57
	(0.37 ± 0.04)	(0.45 ± 0.02)

*o-PM (27 μM) in aqueous solution of 50 mM phosphate buffer, pH 7.0, 22°C.
‡Amplitudes are shown in parentheses.
§Continuous light.

~50°C. The dependence of the rate constants on the temperature is very similar in both crystal forms. The kinetic isotope effect observed in the decay of the M intermediate is ~1.7, which is similar to that found in the hexagonal form. In both cases it can be attributed to the difference in the zero-point energy of the N-H bond vibration.

Immobilization of Bacteriorhodopsin in the Orthorhombic Purple Membrane

The domains of protein-protein interactions are different in the hexagonal and the orthorhombic crystalline arrays of purple membrane. Thus protein-protein interactions could be of different magnitude in the two crystalline forms of purple membrane. Since it was shown that bR is completely immobilized within h-PM (12), we have studied whether a possible weakening of protein-protein interaction would lead to a slow rotational mobility of bR or the retinal chromophore in the o-PM. In the photoselection studied of rotational mobility of bR, we have employed thin o-PM layer on a glass slide, as done in the study of h-PM (12). We have measured the transient dichroism of the M → bR transition, since the slow relaxation time of M in the o-PM enabled us to study the rotational mobility in a time scale of minutes. The transient dichroism in the decay kinetics of the M-bR process is shown in Fig. 7 *a*.

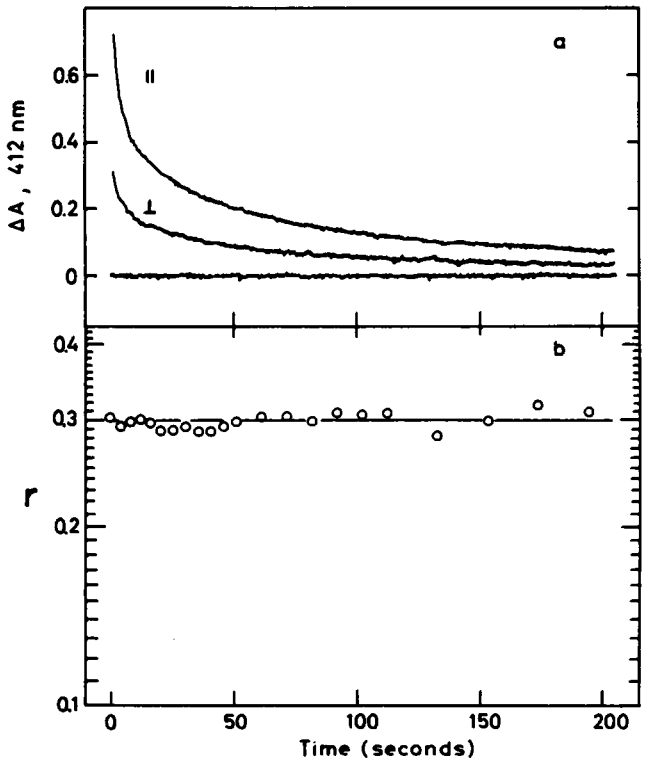

FIGURE 7 (*a*) The transient dichroism in the decay kinetics of M measured in a thin layer of o-PM (equilibrated with 46% relative humidity) at 41°C is illustrated. The axis of both the exciting and the analyzing light formed an angle of 45° with the glass slide plane; (*b*) time dependence of the anisotropy parameter $r(t)$ as calculated from *a*.

Analysis of the data shows that the anisotropy factor, $r(t)$, remains constant over 3.5 min (Fig. 7 *b*), demonstrating the complete immobility of the bR molecule within the o-PM. The results indicate that although protein-protein interactions occur through different parts of the bR molecules, they are strong enough to maintain the complete immobilization of the bR in the o-PM even at 41°C. Furthermore, these findings suggest that the direction of the transition moment of the retinal in the M state does not change upon decay back to the initial bR state. The high anisotropy factor of 0.3 obtained for o-PM is the same as the one found for the h-PM. This suggests that in both crystalline structures the direction of the transition moment of the retinal chromophore is at a similar angle to the plane of the membrane.

Cooperativity in the Photocycle of Orthorhombic Purple Membrane

Since strong interprotein interactions play a key role in the modulation of the photocycle kinetics of bR in the h-PM (6), the existence of similar kinetic coupling in the o-PM was investigated. We have measured the decay kinetics of M from the different photostationary concentration levels back to the equilibrium state (bR). The decay kinetics were fitted by a sum of two exponentials yielding two apparent relaxation time constants. The relaxation time constants were dependent on the (M)/(bR) ratio, as is demonstrated by Tables II and III, suggesting that the decay rates of a bR molecule depend on the conformational state of its nearest neighbors. Though the decay kinetics gave a fit by a sum of two exponentials, the fitting itself was only moderately good, as is demonstrated in the initial decay difference between original and fitted relaxations (Fig. 8). This would be expected if the two fitted apparent

TABLE II
DECAY KINETICS* FROM DIFFERENT PHOTOSTATIONARY LEVELS OF M^{412}

$[M^{412}]/(bR^{564}]$§,‖	τ_2	τ_1
	ms	*ms*
5%	12,967 ± 768	754 ± 22
	(0.54 ± 0.01)‡	(0.36 ± 0.1)
10%	10,762 ± 512	630 ± 30
	(0.43 ± 0.01)	(0.45 ± 0.01)
19%	9,554 ± 203	470 ± 7
	(0.33 ± 0.01)	(0.53 ± 0.01)
30%	8,210 ± 231	380 ± 8
	(0.27 ± 0.01)	(0.59 ± 0.01)
44%	7,495 ± 203	321 ± 14
	(0.22 ± 0.01)	(0.64 ± 0.02)

*o-PM (27 μM) in aqueous solution of 50 mM phosphate buffer, pH 7.0, 6°C.
‡Amplitudes are shown in parentheses.
§Using extinction coefficient of 59,000 (M^{-1}, cm^{-1}).
‖Using a ratio of 0.6 between the difference extinction coefficient of M^{412} and extinction coefficient of bR^{560}.

TABLE III
DECAY KINETICS* FROM DIFFERENT PHOTOSTATIONARY LEVELS OF M^{412}

$[M^{412}]/[bR^{564}]$§,‖	τ_2	τ_1
	ms	*ms*
3%	41,421 ± 7,483	5,239 ± 1,020
	(0.53 ± 0.04)‡	(0.37 ± 0.03)
12%	34,842 ± 2,411	3,326 ± 168
	(0.37 ± 0.01)	(0.45 ± 0.01)
27%	23,654 ± 673	1,291 ± 75
	(0.26 ± 0.01)	(0.50 ± 0.01)
42%	21,253 ± 730	801 ± 24
	(0.21 ± 0.01)	(0.55 ± 0.02)

*o-PM (26 μM) in deuterated aqueous solution of 50 mM phosphate buffer, pH 7.0, 7°C.
‡Amplitudes are shown in parentheses.
§Using extinction coefficient of 59,000 (M^{-1}, cm^{-1}).
‖Using a ratio of 0.6 between the difference extinction coefficient of M^{412} and extinction coefficient of bR^{560}.

relaxation time constants were a combination of sequential and parallel intrinsic relaxation time constant, as in the cooperativity model of the photocycle for h-PM (6). Thus, the results may support the existence of the kinetic coupling between neighboring molecules in the o-PM although the degree of coupling as compared with that existing in the h-PM cannot be determined at this stage.

CONCLUSIONS

Studying the photocycle of bR when packed in two different crystalline forms offers a unique opportunity to examine the molecular packing mode-function correlation of bR in purple membrane. The two different packing arrangements introduce protein-protein interactions through different helical domains of neighboring bR molecules. Thus the effect of the orthorhombic packing mode on the function of bR is presented through spectroscopic and kinetic analysis of the photocycle.

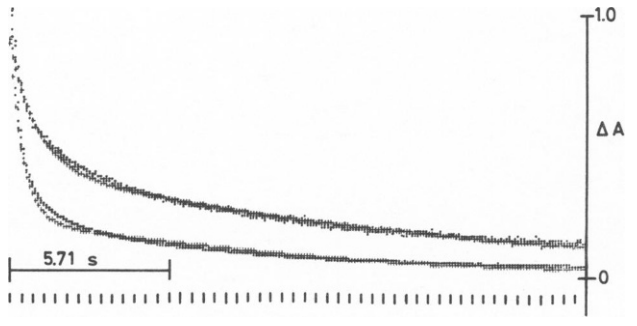

FIGURE 8 The decay kinetics from different photostationary levels of M in aqueous suspension of o-PM (50 mM phosphate buffer, pH 7.0) at 6°C is illustrated: curve *1 (upper)*, decay from photostationary state of A_{412} = 0.420; curve *2 (lower)*, decay from photostationary state of A_{412} = 0.092. Both initial amplitudes of the two decays were normalized. Dots and crosses indicate original data points and best fit data points, respectively.

The data presented show that the basic spectral characteristics of the photocycle are preserved in the o-PM as compared with the h-PM. It supports the notion that the different conformational states of bR in the photocycle are independent of the degree and the mode of aggregation and are rather a characteristic of a single protein molecule. The packing of bR into a crystalline state offers a possible way of modulating the photocycle kinetics. Indeed it manifests itself in slowing the decay of the M intermediate by more than an order of magnitude in the orthorhombic lattice. Moreover, the absence of the 0 intermediate in the o-PM is another consequence of the modified protein-protein interaction. In both crystalline forms the decay kinetics of the M intermediate is dependent on light intensity and duration of the light pulse. These findings cannot be attributed to a delocalized surface membrane phenomena connected with a vectorial pumping (e.g., accumulation of excess of proton at one interface of the purple membrane sheet as compared with the other one) since bR molecules are oriented in opposite directions within o-PM. We suggest that the light intensity dependence of the M decay arises from cooperativity between bR molecules in both o-PM and h-PM.

Support by the Volkswagenwerk Foundation is gratefully acknowledged.

Received for publication 11 October 1983 and in final form 20 August 1984.

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