Biochimica et Biophysica Acta, 546 (1979) 106—120

BBA 47641

A LOW TEMPERATURE INVESTIGATION OF THE INTERMEDIATES OF THE PHOTOCYCLE OF LIGHT-ADAPTED BACTERIORHODOPSIN

OPTICAL ABSORPTION AND FLUORESCENCE MEASUREMENTS *

ARND N. KRIEBEL [a], TOMAS GILLBRO [b,**] and URS P. WILD [c]

[a] *Ciba-Geigy Photochemie AG, CH-1700 Fribourg (Switzerland)*, [b] *The Swedish Research Councils' Laboratory, Studsvik, Fack, S-611 01 Nyköping (Sweden), and* [c] *Laboratorium für Physikalische Chemie, Eidgenössische Technische Hochschule, CH-8092 Zürich (Switzerland)*

(Received April 6th, 1978)
(Revised manuscript received August 18th, 1978)

Key words: Bacteriorhodopsin; Fluorescence; Photocycle; Purple membrane; (Halobacterium halobium)

Summary

Optical absorption and emission measurements have been made on samples of light-adapted purple membrane of *Halobacterium halobium* at temperatures ranging from 77 K to room temperature. As a result of these experiments a set of equations is given which describes thermal and photochemical reactions interrelating various intermediates of the reaction cycle of the chromophore of light-adapted bacteriorhodopsin (BR). Further some specific problems connected to these intermediates have been investigated. Thus the room temperature emission spectrum of bacteriorhodopsin has been found to exhibit a Stokes shift of 3430 cm^{-1} only, if low excitation intensities are used. The recently detected intermediate P-BR can be shown to convert thermally into bacteriorhodopsin following a first-order decay with the activation energy $\Delta E = 2.4 \pm 0.2$ kcal/mol. The thermal decay of K-BR consists of two exponentials if measured on purple membrane suspensions in a mixture of H_2O and glycerol (1 : 1, v/v). A simple procedure is given for trapping the intermediate L-BR at 170 K in a very pure form. M-BR is shown to consist of two species, M_I-BR and M_{II}-BR. They are characterized by similar optical absorption spectra but different thermal stability. Further the oscillator strengths corresponding to the long wavelength absorption bands of the intermediates bacteriorhodop-

* Presented in part at the annual meeting of the Swiss Chemical Society in Bern, Switzerland, on 8 October 1977.

** To whom reprint requests should be sent. Present address: Department of Physical Chemistry, Umeå University, S-901 87 Umeå, Sweden.

sin, K-, L-, M_I- and M_{II}-BR have been calculated. They have been discussed with respect to the question which of the corresponding absorption spectra show the characteristics of isomerism of the chromophore or simply solvatochromism.

Introduction

Optical absorption spectroscopy has been proven to be a most powerful tool for the investigation of the light-induced reaction cycle of the chromophore of light-adapted bacteriorhodopsin (BR) of *Halobacterium halobium*. Starting the reaction cycle by means of a short pulse of actinic light and recording subsequent transient spectral changes has yielded evidence for at least four thermal intermediates called K-, L-, M- and O-BR [1—6]. In later experiments evidence has been furnished that M-BR might consist of two different components [7,8]. However, their relation to each other was not well defined. Emission measurements have further indicated the existence of a compound, P-BR, at 77 K which gives rise to a strong fluorescence [9]. In the course of earlier experiments we have found that the potential capabilities of optical measurements of this kind are far from exhausted. In particular, if one abandons the technique of transient absorptions at room temperature and uses a technique of trapping the intermediates at low temperatures one can study several features of the reaction cycle in detail [1,10—12].

In this paper we report and discuss results we have obtained by using this technique of trapping intermediates.

Experimental

The purple membrane fragments for our experiments were prepared from *H. halobium* (strain R_1M_1) according to the method described by Oesterhelt and Stoeckenius [13]. For the absorption measurements suspensions of purple membrane in H_2O/glycerol mixtures (1 : 1, v/v) with a protein concentration of 0.6 mM were used. The absorption cell consisted of two quartz plates separated by a 0.2 mm teflon spacer. After filling in a drop of the purple membrane suspension between the quartz plates this sandwich-like construction was clamped together and inserted into an Oxford Instruments cryostat DN 704. The cryostat itself was mounted in a Beckman spectrometer (Acta II). It is worth mentioning the high optical quality of these samples. They developed practically no cracks down to 110 K and only a few cracks upon cooling down to 77 K. They show very little light scattering as can be seen from the low absorbance in the region of 21 000 cm^{-1} in Fig. 5a. The various irradiations of the sample have been performed within the mounted cryostat by means of a 1 kW tungsten lamp. Specific wavelengths were selected using interference filters with an average spectral band width of $\Delta\lambda$ = 30—50 nm. The light was directed into the mounted cryostat by means of mirrors.

For the emission spectra less concentrated suspensions (~30 μM) in doubly distilled water were used. The suspensions were then filled into quartz tubes, degassed and then measured at 77 K mounted in an ordinary suprasil dewar

filled with liquid nitrogen. Spectra were taken as described elsewhere [9]. The emission lifetimes were measured by means of a single photon counting fluorimeter [14].

The specific treatment we used to trap intermediates of the reaction cycle will be given for each of the species separately. The nomenclature adopted for the intermediates is that used by Lozier et al. [1] but we omit the superscript LA (light adapted) because we have used light-adapted (LA) samples exclusively. Further we usually add -BR as a suffix. Thus K of ref. 1 will be K-BR in our notation.

Results

The technique of trapping intermediates has allowed us to investigate problems connected with the species bacteriorhodopsin, P-, K-, L-, M_I- and M_{II}-BR. Fluorescence measurements have been confined to bacteriorhodopsin, P- and M_{II}-BR. So far no fluorescence has been observed from K- and L-BR.

The room temperature emission of bacteriorhodopsin

In a first experiment we have measured the emission and excitation spectrum of bacteriorhodopsin at room temperature. In the literature [15] this emission of bacteriorhodopsin has been characterized by a Stokes shift of 4850 cm^{-1}. This appears to be anomalously large because the absorption bandwidth of bacteriorhodopsin at room temperature in only 3430 cm^{-1} (full width at half maximum). The emission spectrum from which this result was derived was obtained by laser excitation (1—10 mW, the area is not given in ref. 15). From this result it was concluded that the emitting state is different from that state which gives rise to the strong absorption of bacteriorhodopsin around 570 nm. In Fig. 1 we show our results as obtained from aqueous suspensions of purple membrane using low intensity excitation (50 $\mu W/cm^2$). The luminescence spectrometer has been described in ref. 16.

Spectrum 2 is the excitation spectrum observed at λ_{em} = 700 nm. Spectrum 1 is the absorption spectrum of bacteriorhodopsin. The good fit of the spectra 1 and 2 shows that the emission which we observe is caused by the excitation of bacteriorhodopsin. Spectrum 3 gives the emission which is observed when bacteriorhodopsin is excited with light of 570 nm (50 $\mu W/cm^2$). This spectrum has also been corrected for the fluorimeter response. It peaks at 714 nm. Spectrum 4, on the other hand, is obtained when bacteriorhodopsin is excited with light of 573 nm (~3 W/cm^2) using an argon ion laser pumped Rhodamin 6G dye laser as excitation source. Spectra 2, 3 and 4 have been corrected for the fluorimeter response (quanta/cm^{-1}). We note that there is a redshift of the emission by about 400 cm^{-1} when high intensity is used instead of a weak light source. This redshift is most likely due to the emission from P-BR (see below). The set of spectra 2 and 3, as obtained from low intensity excitation do not show an anomalous large Stokes shift. The peak separation of the spectra 2 and 3, 3540 cm^{-1}, is comparable with the width of the bacteriorhodopsin band, 3430 cm^{-1}. For a Franck-Condon envelope like that of the absorption band of bacteriorhodopsin this relation between Stokes shift and absorption band width is considered to be normal.

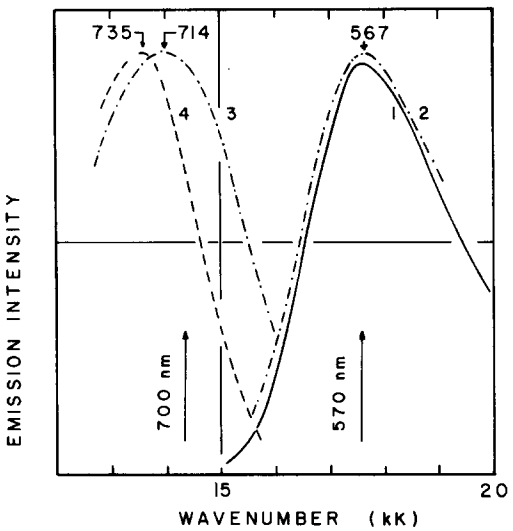

Fig. 1. Room temperature spectra of bacteriorhodopsin in H_2O. 1, absorption; 2, excitation; 3, emission (both excited with low light intensities); 4, laser excited emission spectrum. Peak positions are given in nm. 1 kK = 1000 cm^{-1}.

The thermal decay of K-BR

A sample of purple membrane at 150 K containing the chromophore mainly in the L-BR form (for the preparation see below) shows a photochemical reaction upon irradiation with light of 490 nm. The spectrum of the photoproduct is redshifted and indistinguishable from that of K-BR in the wavelength region where the spectra of K-BR and bacteriorhodopsin do not overlap. At 180 K this photoproduct decays thermally on a time scale of minutes into L-BR. Besides the desire not to introduce more intermediates into the reaction cycle of bacteriorhodopsin than verified by experimental evidence these two properties mentioned before lead us to assume that 490 nm light converts L-Br at least to a major fraction back to K-BR (see also ref. 11). In Fig. 2a we present the decay of the absorption at 630 nm and 180 K after the actinic 490 nm light converting L-BR back into K-BR has been shut off. Since at 630 nm any contribution of L-BR to the absorption is negligible this curve monitors the thermal decay of K-BR. The semilogarithmical plot of Fig. 2b reveals a superposition of two exponentials.

The thermal decay of P-BR

In ref. 9 we showed that strong 570 nm laser excitation of a sample of light-adapted purple membrane after cooling down to 77 K in the dark increased the luminescence intensity observed at 670 nm by a factor of 3. This was attributed to the photolytical build-up of the intermediate P-BR. Using weak 570 nm light (50 $\mu W/cm^2$) allow us even to follow this build-up from a much earlier stage. In the course of such an actinic irradiation the 670 nm luminescence intensity reaches a saturation level which is stronger than the initial level by a factor of at least 50. The luminescence at 670 nm and 77 K can, therefore, be attributed almost entirely to P-BR. At 77 K P-BR is stable for about 1 h. At

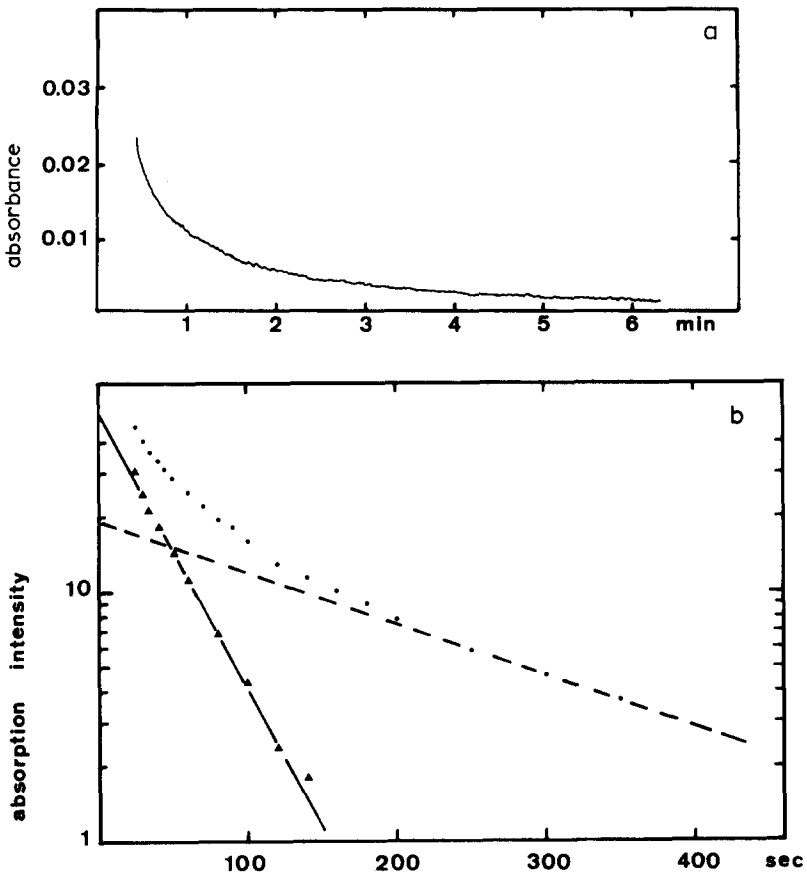

Fig. 2 (a) The decay of the absorption of K-BR at 630 nm (180 K). (b) Resolution of the thermal decay of K-BR (see a) into two exponential components.

90 K, however, it decays thermally on a time scale of minutes. This follows from the decay of the 670 nm luminescence which can be monitored using very weak 570 excitation intensities. In Fig. 3a this decay of the luminescence intensity of P-BR is plotted on the semilogarithmical scale for the temperatures 97 K and 86.3 K. The decay proves to be exponential in the observed time interval of 500 s. In Fig. 3b the slopes of such decay curves are plotted for the temperatures 97, 91, 88.3 and 86.3 K. A linear regression analysis applied to the four points in Fig. 3b yields values for the activation energy and the frequency factor of $\Delta E = 2.35 \pm 0.2$ kcal/mol and $A = 1.26 \cdot 10^{(3\pm0.5)}$ s^{-1}. From these data one calculates for P-BR the lifetimes of $\tau(298) = 42$ ms and $\tau(77) = 63$ min at 298 K and 77 K, respectively. Because of the large error in A these lifetimes are correct within a factor of 3 only.

A procedure for trapping L-BR

If one cools a sample of light-adapted purple membrane suspended in a mixture of glycerol/H_2O (1 : 1, v/v) down to 170 K in the dark one finds the spectrum 1 of Fig. 4. It represents pure bacteriorhodopsin. Successive irradia-

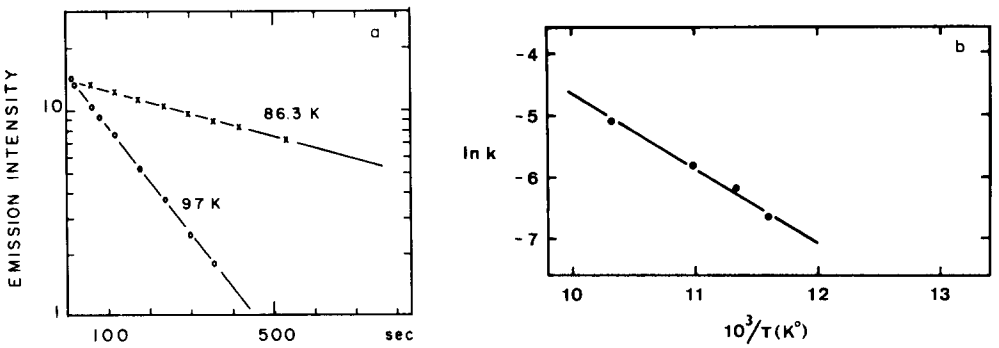

Fig. 3. (a) Thermal decay of the P-emission intensity at 86.3 K and 97 K. (b) Arrhenius plot of the P-emission decay for the temperatures 97, 91, 88.3 and 86.3 K.

tions with 650 nm light ($\Delta\lambda$ = 50 nm, see horizontal bar in Fig. 3, 50 W/cm^2) for 1, 2 and 11 min, respectively, yield the spectra 2, 3, and 4 of Fig. 4. Continued irradiation does not induced further observable spectral changes. There is an isosbestic point at 538 nm. This indicates that only two components contribute to the absorption. The final spectrum (4) peaks at 550 nm. Since this position agrees very well with the results from other authors [1] our procedure seems to yield very pure L-BR.

The resolution of M-BR into M_I- and M_{II}-BR

The experimental procedure which led to the resolution of M-BR into two species will be described in some detail. The spectroscopic evidence for these two intermediates is presented in Fig. 5a. Spectrum 1 in Fig. 5a represents bacteriorhodopsin at 130 K. It has been added for comparison. Spectrum 2

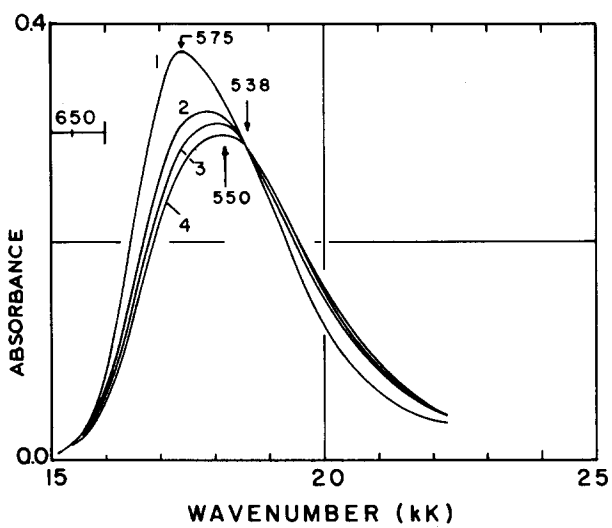

Fig. 4. The temperature-assisted photochemical conversion of bacteriorhodopsin into L-BR at 170 K with light of 650 nm (50 mW/cm^2). Spectrum 1, bacteriorhodopsin, spectra 2, 3 and 4 correspond to intervals of irradiation of 1, 2 and 11 min, respectively. An isosbestic point is found at 18 575 cm^{-1} (538 nm). The horizontal bar indicates the bandwidth of the actinic light.

represents a sample of purple membrane which has been cooled down to 130 K under simultaneous irradiation with light of 550 nm ($\Delta\lambda$ = 50 nm, 100 W/cm^2). Attributing the residual absorption in the region of 550 nm mainly to L-BR one estimates that about 60% of the chromophores have been converted into M-BR which gives rise to the structured absorption around 414 nm. If one

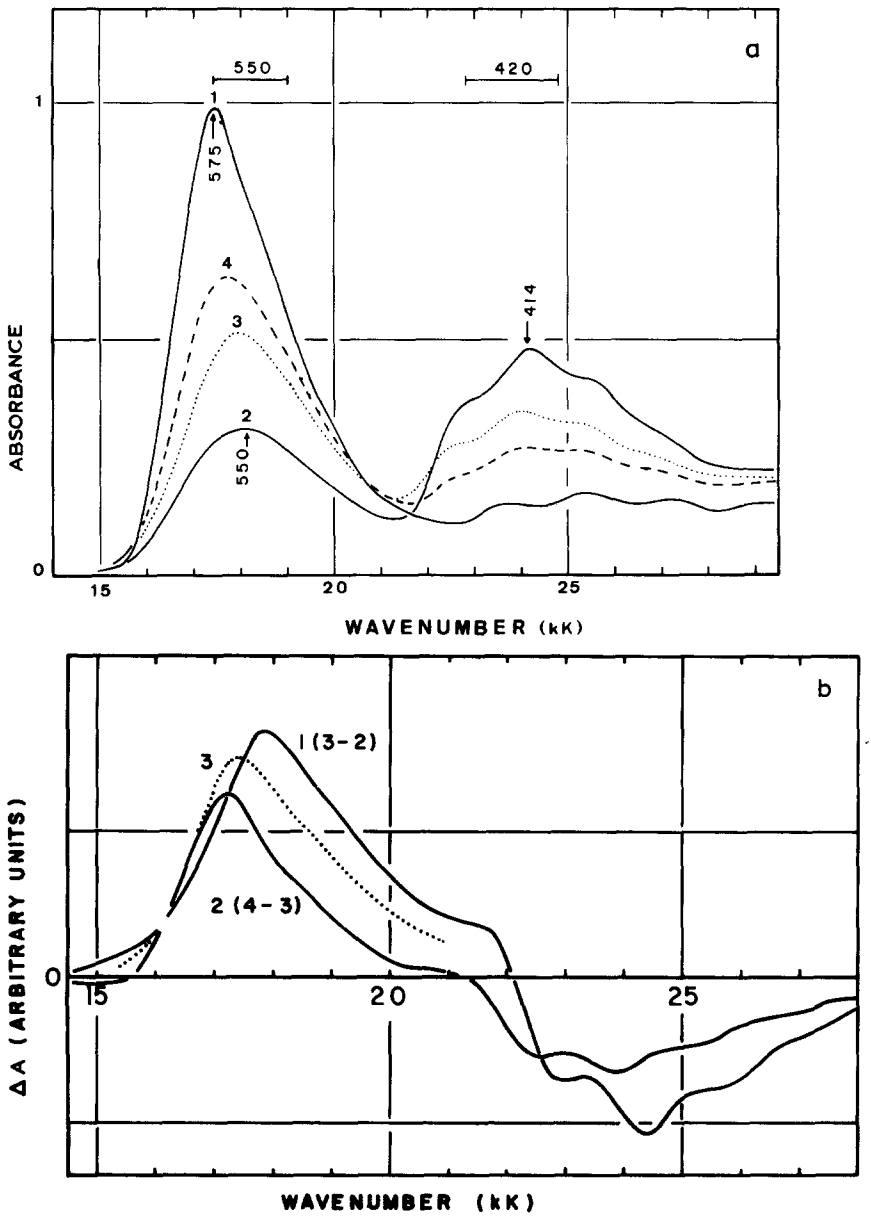

Fig. 5 (a) Photochemical and thermal effects of a bleached sample containing M_I-BR in an initial stage at 130 K (see text).1, bacteriorhodopsin; 2, bleached sample; 3, result of 420 nm irradiation; 4, effect of following warming up to 160 K for 10 min and cooling back to 130 K. The horizontal bar indicates the bandwidth of the actinic light. (b) The difference spectra as obtained from a.

warms up the sample to 160 K for 10 min and cools it back to 130 K avoiding any actinic light spectrum 2 can be reproduced identically. No thermally induced spectral changes are observed at this point. This indicates that the trapped M-BR species is thermally stable at least up to 160 K. If one irradiates the sample at 130 K with light of 420 nm ($\Delta\lambda$ = 35 nm, 40 W/cm^2) for 1 min one obtains spectrum 3. A photochemical conversion has occurred. The reaction product absorbs just in the spectral region where the other known intermediates of the reaction cycle absorb as well. If the warming up procedure to 160 K is repeated one obtains spectrum 4. We observe that at this time there is a thermally induced decrease of absorption in the spectral region of 414 nm paralleled by an increase of absorption in the red region at 570 nm. From this we conclude that the photochemical reaction of the initially prepared M-BR has led to a red absorbing species as well as to another blue absorbing species. This second blue absorbing species decays thermally at 160 K which is in contrast to the thermal stability of the initially trapped M-BR. The species being stable at 160 K we call M_I-BR and the other M_{II}-BR. The spectral properties of these photochemical and thermal products are best illustrated by the difference spectra which can be derived from the spectra of Fig. 5a. They are displayed in Fig. 5b. We identify the blue part of spectrum 2 in Fig. 5a as the absorption of M_I-BR since it does not contain a thermally unstable component. Taking the blue part of the difference spectrum 2 of Fig. 5b as the absorption of M_{II}-BR it follows that the absorption spectrum of M_{II}-BR is redshifted with respect to the absorption of M_I-BR about 3 nm. Further we note that the red-absorbing photoproducts of M_I-BR seem to be distinct from those products which are formed from M_{II}-BR by thermal decay (see spectra 1 and 2 in Fig. 5b). The spectrum of light-adapted bacteriorhodopsin (spectrum 3 in Fig. 5b) has been added for comparison.

An attempt to detect differences in the emission properties of M_I- and M_{II}-BR was not successful. Using a single photon counting fluorimeter [14] (modelocked argon ion laser, 457.9 nm pulses, pulse width 500 ps, pulse separation 8.8 ns, peak power 3 W) we have found that the emission of a bleached purple membrane sample containing mainly M_{II}-BR and possibly some M_I-BR yields a fluorescence with the following properties. The fluorescence decay does not occur according to a single exponential but can be fitted by two decay times, $\tau_1 = 2.5 \pm 0.5$ ns and $\tau_2 = 0.2 \pm 0.1$ ns. Therefore time resolved emission spectra can be taken. The short lived emission spectrum (τ_2 = 0.2 ns) shows the three peaks characteristic for the M-BR species [17]. The long-lived emission spectrum (τ_1 = 2.5 ns) shows only little structure and belongs most likely to neither M_I- nor M_{II}-BR. Thus these results do not yield further criteria to distinguish M_I- and M_{II}-BR. More details about these experiments will be published elsewhere.

Photochemical and thermal reactions relating various intermediates

Hints on the nature of the photochemical products of M_I-BR can be derived from Fig. 6a. Spectrum 2 of Fig. 6a is obtained by cooling a sample of light-adapted bacteriorhodopsin once again down to 110 K under simultaneous irradiation with light of 550 nm. In order to remove K-BR, which might be trapped at these temperatures, the sample is irradiated with red light of 650 nm

(50 W/cm^2) for 1 min. Spectrum 2 is taken. Subsequent irradiation of the sample with light of 420 nm for 1 min and afterwards with light of 650 nm for another minute yields spectrum 3. Further irradiation with light of 420 nm for 3 min leads to spectrum 4. It can be shown that the photochemical conversion slows down and reaches a state of saturation.

The last conversion can be reversed almost completely by 650 nm irradiation as indicated by spectrum 5. The information one can draw from these experiments is most important. They are visualized from the corresponding difference

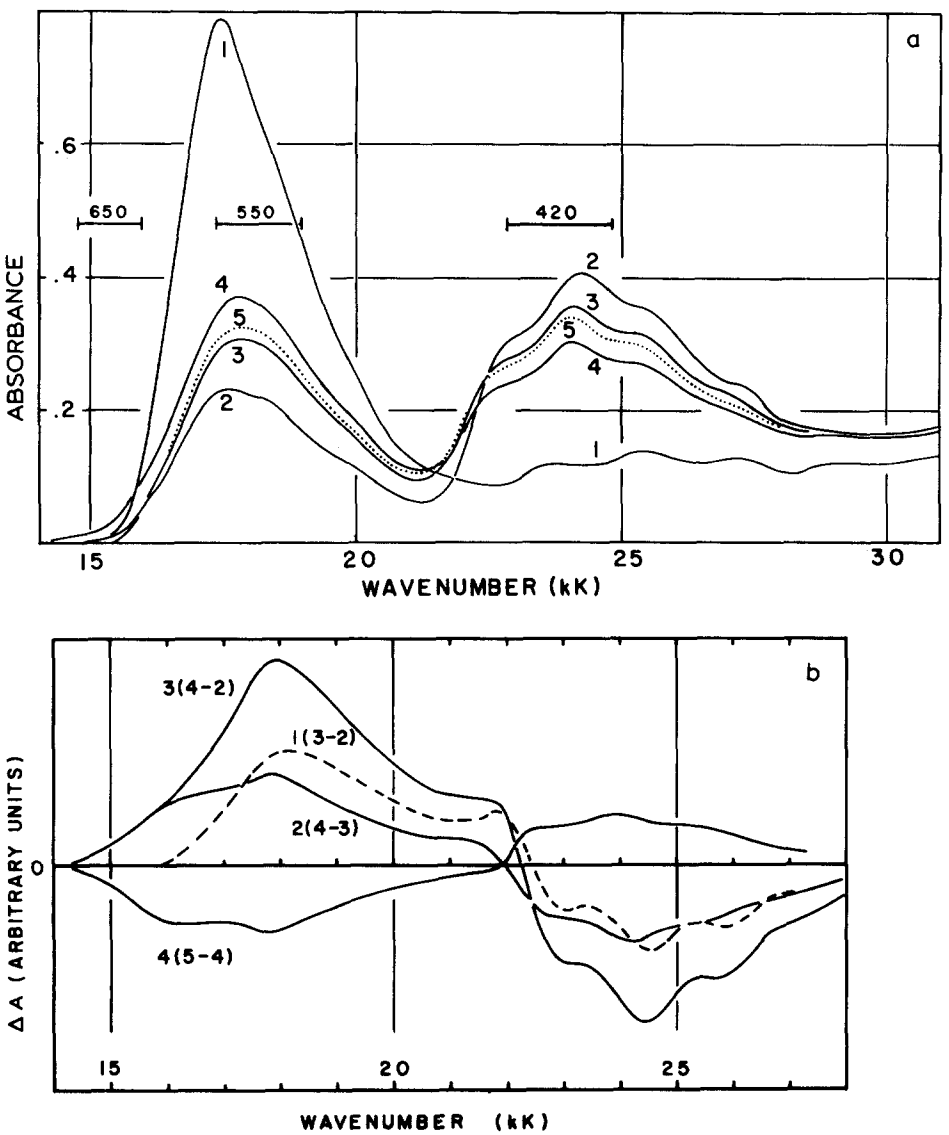

Fig. 6. (a) The partial reversibility of the photoreaction of M_I-BR at 130 K. 1, bacteriorhodopsin; 2, bleached sample; 3, result of 420 nm irradiation for 1 min and following 650 nm irradiation for 1 min; 4, result of additional 420 nm irradiation for 3 min; 5, subsequent 650 nm irradiation. The horizontal bars represent the bandwidth of the actinic light. (b) The difference spectra as obtained from a.

spectra in Fig. 6b. We note that two photoproducts absorbing in the red are formed in the course of the photoconversion of M_I-BR into M_{II}-BR. One of them has an absorption spectrum which resembles most closely that of L-BR (compare spectra 1 in Fig. 6b and 4 in Fig. 8). This product is formed in easily observable amounts only during the first 420 nm irradiation when the initial M-intermediate is known to be M_I-BR. the second photoproduct, X-BR, absorbs more to the red (see spectrum 2 in Fig. 6a) and can be back converted to an M-intermediate (see spectrum 4 in Fig. 6b). Spectrum 6 in Fig. 7a confirms that the M-intermediate can once again react photolytically to X-BR. This establishes the existence of a photo-reversible reaction between X-BR and the two M-species. Spectrum 2 in Fig. 6b indicates that during blue irradiations (420 nm) following the first one the red-absorbing product X-BR is by far the

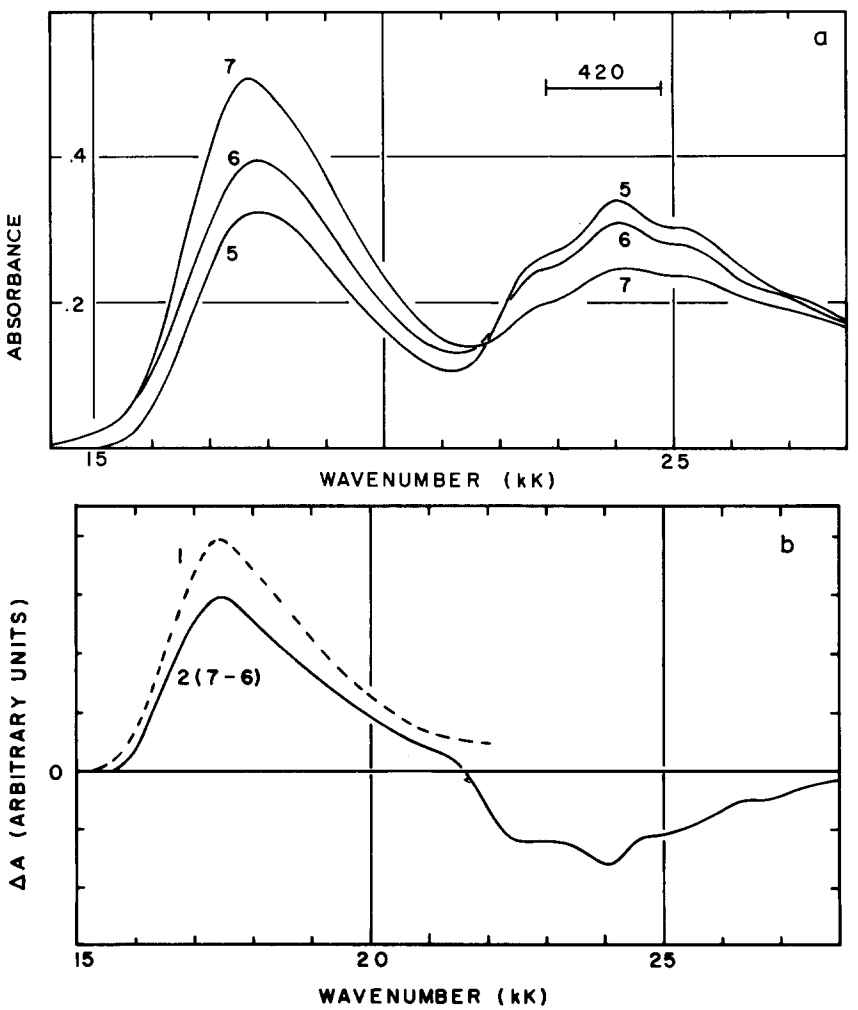

Fig. 7. (a) Continuation of the experiment depicted by Fig. 6a. Spectrum 5, repeated from Fig. 6a; 6, repetition of the 420 nm irradiation; 7, result of warming up to 160 K. (b) The difference spectra as obtained from a.

dominating photoproduct. This is in contrast to the result of the first 420 nm irradiation where a large amount of L-BR is formed (spectrum 1). Therefore, it is reasonable to consider X-BR to be the product of a photolytical reaction of M_{II}-BR. The reversibility of this photoreaction does not mean necessarily that 650 nm light converts X-BR back to M_{II}-BR. A return to M_I-BR cannot be ruled out. The answer to this question is determined by the interpretation of the slight deviation of spectrum 4 from being a perfect mirror image of spectrum 2.

From Fig. 7a it can be seen what happens if the experiment of Fig. 6a is continued by 420 nm irradiation for 1 min. The reversible photoreaction of Fig. 6b is once again confirmed by spectrum 6. Spectrum 7 shows the result of a following warming up procedure to 160 K. But if we take now the difference spectrum 7—6 as depicted in Fig. 7b we note that at this time the product of the thermal decay fits the absorption of pure bacteriorhodopsin as given by spectrum 1.

The optical absorption parameters of bacteriorhodopsin, K-, L-, M_I-, M_{II}-BR

In order to get a parameter which is sensitive to the molecular structure of the intermediates we have calculated the oscillator strengths f and the transition dipole moments μ_{eg} corresponding to the long wavelength transitions of the intermediates bacteriorhodopsin, K-, L-, M_I- and M_{II}-BR. We have used our own spectra (see Fig. 8) as well as spectra of other authors. For example, we have not succeeded to trap pure K-BR in order to measure its spectrum directly. For this reason we have included in Fig. 8 the K-BR spectrum as calculated by Lozier and Niederberger [10]. The spectrum 5 of M_I-BR represents the blue part of spectrum 2 in Fig. 5a. The spectrum 6 of M_{II}-BR corresponds to the blue part of the difference spectrum 2 in Fig. 7b. In Table I we have summarized the following spectral data of the intermediates bacteriorho-

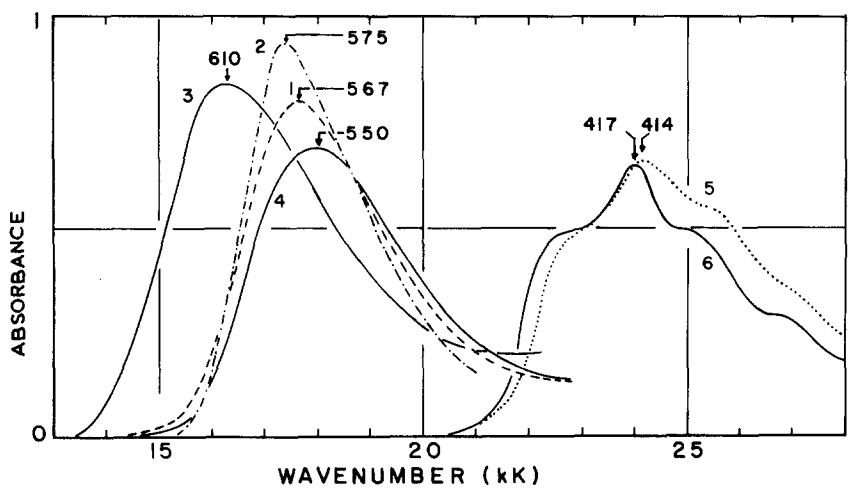

Fig. 8. The absorption spectra of intermediates of the reaction cycle in H_2O/glycerol (1 : 1, v/v) at 130 K if not otherwise specified. 1, bacteriorhodopsin (room temperature); 2, bacteriorhodopsin; 3, K (77 K); 4, L-BR; 5, M_I-BR; 6, M_{II}-BR. Peak positions are given in nm.

TABLE I

OPTICAL ABSORPTION PARAMETERS OF INTERMEDIATES OF THE REACTION CYCLE

Intermediate [a]	Solvent [b]	T (K)	ϵ_{max} (10^{-4} l/mol cm)	λ_{max} (nm)	$\tilde{\nu}_{max}$ (cm^{-1})	nf [c]	$\sqrt{n}\,\mu_{eg}$ [c] (D)	Ref.
BR l.a.	A	298	6.3	568	17 606	0.94	10.2	
BR l.a.	B	298	6.3	567	17 637	0.99	10.7	24
BR d.a.	B	298	5.6	559	17 889	0.93	10.3	24
BR l.a.	A	130	7.5	575	17 391	0.97	10.7	
BR l.a.	?	77	7.9 [d]					10
P-BR	B	77		597 [e]	16 750			
K-BR	?	77	7.1 [d]	610	16 393	1.34	13.0	10
L-BR	A	130	5.5	550	18 182	0.93	10.3	
L-BR	?	77	5.8	546	18 315	1.15	11.1	10
M_I-BR	A	130	5.3	414	24 155	1.06	9.6	
M_{II}-BR	A	130	5.2	417	23 981	0.94	9.1	

[a] l.a. and d.a. stand for light and dark adapted, respectively.
[b] A stands for H_2O/glycerol (1 : 1, v/v) and B stands for H_2O.
[c] n is the refractive index of the solvent.
[d] These extinction coefficients are our numbers.
[e] This wavelength represents the peak position of our P-excitation spectrum [9].

dopsin, P-, K-, L-, M_I- and M_{II}-BR: position of the absorption maximum (cm^{-1} and nm), molar extinction coefficient ϵ(l/mol cm), oscillator strength f, transition dipole moment μ_{eg} (D). Since we do not know what the exact index of refraction, n, is for our glycerol/H_2O mixtures (1 : 1, v/v) the oscillator strengths and the transition dipoles are weighted by n and $\sqrt{n}$, respectively. The oscillator strengths nf in Table I are presumably correct within 5%. One uncertainty results from the lack of knowledge how far to the blue an absorption band has to be considered to calculate f.

Discussion

The most conspicuous interrelations which follow from our experiments can be summarized by the following equations:

$$BR \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} K \underset{h\nu}{\overset{kT}{\rightleftharpoons}} L \overset{kT}{\rightarrow} M_I \tag{1}$$

$$M_I \overset{h\nu}{\rightarrow} M_{II} + L \tag{2}$$

$$M_{II} \overset{kT}{\rightarrow} BR + (Y) \tag{3}$$

$$M_{II} \overset{h\nu}{\rightarrow} X \tag{4}$$

$$X \overset{h\nu}{\rightarrow} M_{II} \text{ or} \tag{5a}$$

$$X \overset{h\nu}{\rightarrow} M_I \tag{5b}$$

$$BR \overset{h\nu}{\rightarrow} P \overset{kT}{\rightarrow} BR \tag{6}$$

The letters *hν* or *kT* on top of the reaction arrows indicate whether the reaction is photolytical or thermal. Eqn. 2 follows from Figs. 5a, 5b, 6a and 6b, Eqn. 3 from Figs. 5b and 7b, Eqn. 4 from Figs. 6a and 6b. Eqns. 5a and 5b describe the two possible back reactions of X as demonstrated by spectrum 4 in Fig. 6b. Eqn. 6 represents our results concerning the nature of the observed fluorescence from samples of light-adapted bacteriorhodopsin in H_2O at 77 K. In Eqn. 3 we have added an intermediate Y in brackets. This is supposed to account for the difference spectrum 2 in Fig. 5b. It does not fit the absorption of bacteriorhodopsin but looks as if a second product absorbing in the red has been produced by the thermal decay. On the other hand one notes from the same spectrum that warming up to 160 K has led to a decrease of absorption around 15 000 cm^{-1}, so in this experiment the distortion of the difference spectrum with respect to that of bacteriorhodopsin might have other reasons than the formation of compound Y. Using red illumination in between as in Fig. 7b obviously eliminates this cause of spectrum distortion. It is possible that X-BR is identical to O-BR, because of the late appearance of these species in the reaction cycle [6] and their red-shifted absorption maxima.

At this point we can respond to a view published very recently after our experiments had been finished. In ref. 18 it is claimed that there exist photolytical equilibria $L \rightleftharpoons L'$ and $M \rightleftharpoons M'$ comparable to that between bacteriorhodopsin and K-BR. In the first case, $L \rightleftharpoons L'$, the authors have not rationalized that L can be back converted by 520 nm radiation first to K-BR and therefore afterwards to bacteriorhodopsin (see Eqn. 1). This accounts for their observed increase in the bacteriorhodopsin concentration without the necessity of introducing a new intermediate L'. In the second case, $M \rightleftharpoons M'$, their stable steady-state concentration of M' does not indicate unambiguously a photoequilibrium but can equally well be explained by our Eqs. 2—5. The reason for the conclusions in ref. 18 can be found in the fact that obviously the photoproducts at low temperatures have not been investigated but only their subsequent thermal decay products at 183 K.

In a next step we discuss the optical parameters of the intermediates. Since only one chemical reaction (deprotonation) is known to occur in the course of the reaction cycle in purple membrane two other possibilities have to be considered to account for the large variety of spectral changes as depicted, e.g. in Fig. 8. First of all the chromophore can suffer conformational changes like twisting or *cis-trans* isomerism around single or double bonds (isomerism). They tend to alter the molecular eigenstates of the chromophore and to change the position, the intensity, and the Franck-Condon structure of the optical absorption bands. Secondly, induced changes of the surrounding of the chromophore might occur (solvatochromism). Solvatochromism usually affects the position of the absorption band alone leaving the oscillator strength and Franck-Condon structure almost unchanged. This last statement is valid whenever the chromophore is sufficiently rigid [19] and mixing of electronic states due to different local electric fields is negligible. Applying this criterion to distinguish between isomerism and solvatochromism to the data in Table I (columns 5—7) and Fig. 8 one concludes that the spectra of K-, M_I- and M_{II}-BR show changes with respect to that of BR which are more complex than expected from simple sovatochromism. Intrinsic changes of the chromophore

have occurred. Comparing the spectral data of M_I-BR and M_{II}-BR, however, one finds that the Franck-Condon structure as well as the oscillator strength of the long wavelength absorption band are conserved. The spectral shift of 3 nm could be explained as solvatochromism. Converting M_I-BR photolytically into M_{II}-BR thus might simply mean to induce changes in the protein structure in the vicinity of the chromophore. These changes alter the local electric field and facilitate the thermal decay of M_{II}-BR into bacteriorhodopsin (see Fig. 7a).

A further point which deserves discussion is P-BR which we showed earlier [9] to be responsible for the strong fluorescence of bacteriorhodopsin samples at 77 K. Our results as described in the present work show that P-BR is formed photolytically from bacteriorhodopsin and decays thermally. We have also found that the saturation concentration of P-BR, which one obtains at 77 K by illuminating the sample with actinic light (50 $\mu W/cm^2$), is independent of the exciting wavelength. These results together with our failure to detect any absorption of P-BR at 77 K leave the following two possibilities open. (a) If the absorption spectrum of P-BR at 77 K is similar to its excitation spectrum, which has a maximum around 597 nm [9], the concentration of P-BR has to be low, i.e. $[P] < [BR] \times 10^{-2}$. (b) If the absorption spectrum of P-BR is similar to that of bacteriorhodopsin, as indicated by recent flash kinetic experiments [6] at ambient temperatures, the concentration of P-BR might even be high.

The strong emission from P-BR in spite of its short fluorescence life time of 15—40 ps [20,21] suggests that the concentration of P-BR is high, however, recent results on all *trans*-retinal have shown that small amounts of photoproducts might contribute significantly to the total emission [22,23].

Finally, we want to discuss the decay of K-BR in glycerol/H_2O. It consists of two first-order reactions (Fig. 2b) just like the decay of L-BR and M-BR in some suspensions, e.g. at $pH > 8$ [6,8]. The simplest explanation for this behaviour is that there are two populations of bacteriorhodopsin, which in neutral aqueous suspensions are indistinguishable but in other solvents react with different rate constants. It might be that the purple membrane structure is distorted by some solvents as indicated by higher speed of rotational diffusion [6].

Acknowledgements

The experimental work reported in this paper was performed in the Laboratory of Physical Chemistry at Eidgenössische Technische Hochschule, Zürich and we want to thank the staff, especially H-P. Good, H-G. Griesser, Dr. A. Holzwarth and P. Müller, for their kind assistance during the course of this work. Dr. U. Müller and Dr. R. Cherry are gratefully acknowledged for providing the purple membrane suspension. Financial support was obtained from the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung and the Swedisch Natural Science Research Council. T.G. is indebted to 'Roche Research Foundation for Scientific Exchange and Biomedical Collaboration with Switzerland' for a research grant, which made a prolongation of his stay at Eidgenössische Technische Hochschule, Zürich possible.

References

1 Lozier, R.H., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 955—962
2 Goldschmidt, C.R., Ottolenghi, M. and Korenstein, R. (1976) Biophys. J. 16, 839—843
3 Dencher, N. and Wilms, M. (1975) Biophys. Struct. Mech. 1, 259—271
4 Kung, M.Chu., Devault, D., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 907—911
5 Chance, B., Porte, M., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 913—917
6 Gillbro, T. (1978) Biochim. Biophys. Acta 504, 175—186
7 Slifkin, M.A. and Caplan, S.R. (1975) Nature 253, 56—58
8 Hess, B. and Kuschmitz, D. (1977) FEBS Lett. 74, 20—24
9 Gillbro, T., Kriebel, A.N. and Wild, U.P. (1977) FEBS Lett. 78, 57—60
10 Lozier, R.H. and Niederberger, W. (1977) Fed. Proc. 36, 1805—1809
11 Litvin, F.F., Balashov, S.P. and Sineshchekov, V.A. (1975) Bioorg. Khim. 1, 1767—1777
12 Litvin, F.F. and Balashov, S.P. (1977) Biofizika 22, 1111—1114
13 Oesterhelt, D. and Stoeckenius, W. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXI, pp. 667—678, Academic Press, New York
14 Wild, U.P., Holzwarth, A. and Good, H.P. (1977) Rev. Sci. Instr. 48, 1621—1627
15 Lewis, A., Spoonhower, J.P. and Perreault, G.J. (1976) Nature 260, 675—678
16 Vo Dinh Tuan, and Wild, U.P. (1973) Appl. Opt. 12, 1286
17 Gillbro, T. and Kriebel, A.N. (1977) FEBS Lett. 79, 29—32
18 Hurley, J.B., Becher, B. and Ebrey, T.G. (1978) Nature 272, 87
19 Liptay, W. (1965) in Modern Quantum Chemistry (Sinanoglu, O., ed.), part III, Academic Press, New York
20 Hirsch, M.D., Marcus, M.A., Lewis, A., Mahr, H. and Frigo, N. (1976) Biophys. J. 16, 1399—1409
21 Alfano, R.R., Yu, W., Govindjee, R., Becher, B. and Ebrey, T.G. (1976) Biophys. J. 16, 541—545
22 Takemura, T., Das, P.K., Hug, G. and Becker, R.S. (1976) J. Am. Chem. Soc. 98, 7099—7101
23 Hochstrasser, R.M. and Narva, D.L. (1977) Photochem. Photobiol. 26, 595—599
24 Becher, B. and Cassim, J.Y. (1976) Biophys. J. 16, 1183—1199