HYDRATION EFFECTS ON *CIS–TRANS* ISOMERIZATION OF BACTERIORHODOPSIN

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1. Introduction

Cis–trans isomerization is the primary photochemical event in the photochemistry of natural and artificial visual pigments [1,2]. Although the retinal–opsin interaction is the underlying constraint for the photochemistry of vision, not much information is available about the role of the opsin in these processes. A possible approach to such a question would be the study of the effects of protein conformational changes on the primary photochemical event. We have adapted such an approach in the study of the *cis–trans* isomerization of bacteriorhodopsin. We present here the reversible effects of hydration changes on the *cis–trans* isomerization of bacteriorhodopsin and demonstrate that the isomerization process is stopped at low hydration states. Furthermore the quantum yield for 13-*cis* → all-*trans* isomerization, is shown to be wavelength independent. It is suggested that 13-*cis* → all-*trans* photoisomerization takes place after complete thermal relaxation in the excited state.

Bacteriorhodopsin can be found in two interconvertible forms [3]: light adapted and dark adapted. Bacteriorhodopsin when kept in dark at 25°C has its longest wavelength absorption maximum at 560 nm, and is defined as dark-adapted bacteriorhopdopsin ($BR^{DA}_{25°C}$). Irradiation of BR^{DA} causes a red shift of the absorption maximum to 570 nm together with an increase of the molar extinction coefficient. This form which is defined as light-adapted bacteriorhodopsin (BR^{LA}), reverts thermally to BR^{DA}. Chemical analyses of the retinal configuration in BR^{LA} have shown [3–6] that it consists of a single isomer, all-*trans* retinal. Thin layer chromatography of retinal extracted from BR^{DA} showed that it consists of a mixture of 13-*cis* and all-*trans* retinal [3]. Later it was reported [4] that the retinal in BR^{DA} is exclusively 13-*cis*. However, using high-pressure liquid chromatography, it has been found [5,6] that retinal extracted from BR^{DA} at 25°C, consist of 50% 13-*cis* and 50% all-*trans* thus confirming the early report. It can therefore be concluded that dark–light adaptation represents isomerization of the 13-*cis* retinal into the all-*trans* configuration. $BR^{all\text{-}trans}$ (BR^{LA}) [7–9] and $BR^{13\text{-}cis}$ [10] undergo separate cycles where 13-*cis* → all-*trans* isomerization is the interconnecting step of the two cycles (fig.1).

2. Materials and methods

Purple membrane fragments were isolated from *Halobacterium halobium* (NRL R_1M_1) [3]. Thin layers of purple membrane were prepared by drying concentrated suspensions of purple membrane in water (pH 7.2) on a glass slide at room temperature

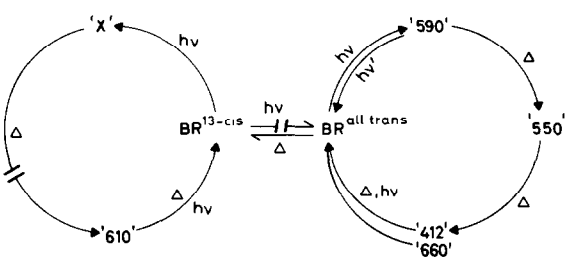

Fig.1. Scheme of $BR^{all\text{-}trans}$ and $BR^{13\text{-}cis}$ cycles and the *cis–trans* interconnecting route of the two cycles. The (⊣ ↦) represent pathways that are blocked on reducing the hydration state of the purple membrane.

and atmospheric pressure. The thickness of the preparations was 1–3 μm, as determined by scanning electron microscopy. Variable hydration of the thin layers was obtained by equilibrating the preparations with various relative humidities produced by saturated salt solutions [11]. The samples were incubated at a specific humidity for 24 h.

The dark–light adaptation was accomplished by irradiating BR^{DA} with a 400 W W–I_2 lamp at wavelengths longer than 500 nm until no further change in the spectra was obtained. The absorption spectra of BR^{LA} and BR^{DA} were measured by a rapid scanning spectrophotometer [12] (Kieler Howaltswerke, Kiel, Germany). The wavelength calibration of the spectra was carried out with a neodynium filter. The determination of the $BR^{DA} \rightarrow BR^{LA}$ quantum yield was performed by irradiating suspended purple membrane fragments in water with a 100 W Mazda mercury lamp, using Corning and Schott glass filter combinations for the different wavelengths. Actinometry was carried out at 365 nm, 405 nm and 436 nm according to Hatchard and Parker [13]. The intensity of the light at 546 nm and 576 nm was compared to the intensity at 365 nm and 436 nm using a YSI Kettering G5A radiometer.

Flash photolytic measurements of '412' were performed with a linearly-polarized light pulse from a rhodamine 6G laser at 585 nm. The samples were excited at right angles to the analyzing light, where the plane of the purple membrane layers formed angles of 45°, both with the exciting light and the analysis light axes.

3. Results and discussion

The absorption spectra of BR^{DA} and BR^{LA} in thin purple membrane layers, equilibrated with 94% humidity, were found to be identical with BR^{DA} and BR^{LA} states of purple membrane fragments suspended in water (fig.2a,b). When

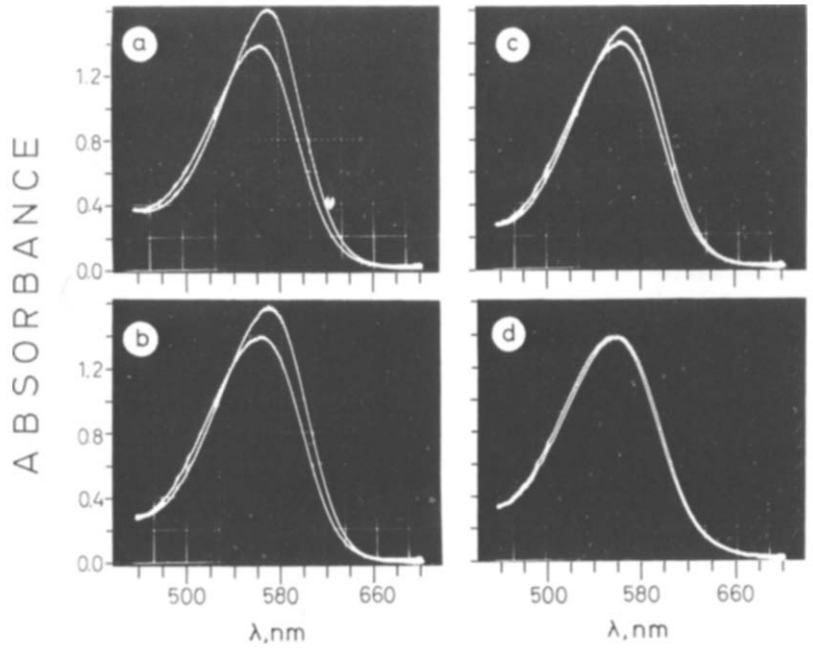

Fig.2. Absorption spectra of BR^{LA} and BR^{DA} states in suspended purple membrane fragments in water and in thin purple membrane layers equilibrated with different relative humidities (22°C). (a) Water. (b) 94% Relative humidity. (c) 43% Relative humidity. (d) 10% Relative humidity.

lowering the hydration state of the thin layers, by changing the relative humidity from 94% to 10%, no shift of the absorption maximum of the BR^{DA} form was observed, although a reduction of up to 10% in the absorption was detected. However, the BR^{LA} state was found to be dependent on the hydration state. Starting with the same initial absorption of BR^{DA}, at the various relative humidities, we obtained different states for BR^{LA}, as the difference spectrum BR^{LA}–BR^{DA} decreased along with lowering the hydration state, until the two states were spectroscopically indistinguishable at 10% relative humidity (fig.2c,d). The same BR^{LA} state was obtained either when irradiating BR^{DA} to the corresponding BR^{LA} at a fixed hydration state, or whether first irradiating BR^{DA} at 94% humidity and then changing the relative humidity to the desired one under constant illumination.

The amplitudes of the difference spectrum BR^{LA}–BR^{DA} measured at 590 nm, at the various humidities, are given in fig.3a. In order to find out whether the observed decrease of the difference amplitude is accompanied by a change in the photochemical yield of the cycle, the amount of '412' intermediate was measured at different humidities, both for the BR^{DA} and BR^{LA} forms (fig.3b).

Since the relative change of the quantum yield for '412' formation can be obtained from the relative change in the amount of '412' formed per flash, (with laser light intensity far from saturation), it emerges from fig.3b curve BR^{DA}, that the formation quantum yield of '412' is almost constant at all humidities. The observed higher amount of '412' formed in the BR^{LA} state as compared with the BR^{DA} state (fig.3b curves BR^{LA}, BR^{DA}) results from '412' being an intermediate of $BR^{all\text{-}trans}$ but not of $BR^{13\text{-}cis}$. Since the concentration of all-*trans* retinal is double in BR^{LA}, as compared with BR^{DA}, an increase by a factor of two is to be expected in the amount of '412' formed per flash in the BR^{LA} state (at 94% relative humidity), as shown by fig.3b. The decrease in the amount of '412' formed per flash in the BR^{LA} state, along with the decrease in the hydration state would therefore suggest enrichment in the 13-*cis* retinal component of the all-*trans*–13-*cis* mixture. This is reflected in the convergence of

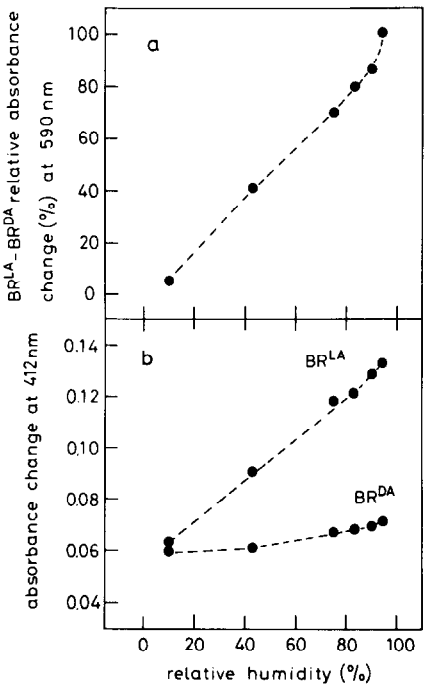

Fig.3. (a) BR^{LA}–BR^{DA} difference absorbance change at 590 nm as a function of relative humidity. (b) Amount of '412' intermediate formed per flash in bacteriorhodopsin at dark- and light-adapted states, in thin purple membrane layers as function of relative humidity.

'412' amplitudes of BR^{LA} and BR^{DA} in accordance with the decrease of the difference absorption spectrum of the two states. It may therefore be concluded that the dark–light adaptation process, corresponding to 13-*cis* → all-*trans* photoisomerization is stopped at low hydration states of the purple membrane. Whereas hydration affects the photochemical route *cis–trans* isomerization, it does not change the thermal decay from the metastable BR^{LA} to BR^{DA} state.

In order to compare the quantum yield for *cis–trans* isomerization with that of the cycle, the quantum yield for dark–light adaptation was measured in purple membrane suspended in water.

Photoisomerization of $BR^{DA} \rightarrow BR^{LA}$ was accomplished by irradiating BR^{DA} at five different wavelengths: 365 nm, 405 nm, 436 nm, 546 nm and 578 nm. The same photostationary state was obtained in each case, independent of the

irradiation wavelength. The photostationary composition obtained when irradiating 13-*cis* $\rightleftharpoons$ all-*trans* system at wavelength λ, is given by

$$\left(\frac{(13\text{-}cis)}{(\text{all-}trans)}\right)_\lambda = \left(\frac{\phi_{t-c}}{\phi_{c-t}}\right)_\lambda \left(\frac{\epsilon_{\text{all-}trans}}{\epsilon_{13\text{-}cis}}\right)_\lambda$$

where ϕ_{t-c} and ϕ_{c-t} are the quantum yields for all-*trans* → 13-*cis* and 13-*cis* → all-*trans* correspondingly and $\epsilon_{\text{all-}trans}$, $\epsilon_{13\text{-}cis}$ are the extinction coefficients. Since the photostationary state was found to be independent at the irradiation wavelength, it suggests that the photostationary state does not depend on $\epsilon_{\text{all-}trans}/\epsilon_{13\text{-}cis}$ ratio. Therefore it can be concluded that $\phi_{t-c} \ll \phi_{c-t}$, thus establishing that the all-*trans* → 13-*cis* is an inefficient photochemical route and can be neglected when compared to that of 13-*cis* → all-*trans*. ϕ_{c-t} was determined to be 0.08 ± 0.02 and was found to be both wavelength and temperature independent (in the range of 365 nm to 578 nm and −3°C to 30°C, correspondingly). The value for ϕ_{c-t} measured here by photostationary methods is in agreement with the value of 0.035 ± 0.07 obtained under flash saturated conditions [14]. The wavelength independence of ϕ_{c-t} indicates that the quantum yield is independent of the vibronic level reached by light absorption. These findings suggest that isomerization takes place after complete thermal relaxation in the excited state and requires no activation energy, like the 11-*cis* → all-*trans* photoisomerization process in rhodopsin [2].

The dependence of the absorption spectrum of the BR^{LA} state on hydration may reflect changes in the photostationary composition of 13-*cis* and all-*trans* isomers. Such changes may be caused either by changes in the efficiencies of competitive routes with the *cis–trans* photoisomerization or may be produced by changes of the ϕ_{c-t}/ϕ_{t-c} ratio. (The possibility that the extinction coefficient $BR^{\text{all-}trans}$ is affected to a different extend than $BR^{13\text{-}cis}$ is less plausible, but cannot be ruled out.) Possible competitive routes with the *cis–trans* photoisomerization are $BR^{\text{all-}trans}$ and $BR^{13\text{-}cis}$ cycles. However no changes in the efficiency of $BR^{\text{all-}trans}$ cycle was observed, since no change in the formation yields of '412' were detected (fig.3b, curve BR^{DA}). Thus assuming no efficiency changes in the competitive routes upon dehydration, it may be suggested that ϕ_{c-t}/ϕ_{t-c} ratio is regulated through conformational changes induced by variation of the hydrophilic interactions through changes in hydration.

The *cis–trans* isomerization connects the cycle of $BR^{\text{all-}trans}$ with that of $BR^{13\text{-}cis}$. However the exact step at which the photoisomerization occurs is still unknown. In a recent study [15] it was shown that the effect of hydration of the $BR^{\text{all-}trans}$ cycle is to decrease the decay rates of '412' and to prevent the formation of '660' at relative humidities lower than 90%. Under similar conditions the formation of '610' in the $BR^{13\text{-}cis}$ cycle is inhibited. The fact that at relative humidities lower than 90% dark–light adaption still occurs suggest that the route of photoisomerization does not pass under these conditions via the '610' intermediate, as was suggested [10]. Therefore it is suggested that photoisomerization occurs via '*x*' or directly from the excited state of 13-*cis*.

Since hydrogen exchange studies have shown that, in rhodopsin, 70% of the peptide hydrogens are freely exposed to solvent water [16], whereas bacteriorhodopsin has about 75% of its peptides internally H-bonded [17], protein–water interaction must be of even greater importance in rhodopsin than in bacteriorhodopsin. Therefore it may be expected that dehydration of rhodopsin would have greater conformational and photochemical consequences than those observed in bacteriorhodopsin, as reflected by the prevention of the *meta*-I → *meta*-II transition [18]. The structural analysis of conformational changes induced by dehydration may illuminate the determining factors in the primary process of vision.

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