ISOMERIC COMPOSITION OF BACTERIORHODOPSIN UNDER DIFFERENT ENVIRONMENTAL LIGHT CONDITIONS

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1. Introduction

Bacteriorhodopsin (BR) is a chromoprotein and the pigment of the purple membrane (PM) of *Halobacterium halobium* [1]. The chromophoric group of BR is retinal, alternatively in either the all-*trans* or 13-*cis* configuration [2]. Other isomers of retinal were not found [3]. BR which contains all-*trans* retinal is called *trans* BR (BR^{568}_{trans}), BR which contains 13-*cis* retinal is called 13-*cis* BR ($BR^{548}_{13\text{-}cis}$). The two BR isomers are interconvertible by means of several pathways. One pathway does not require light ('dark adaptation'); the others are initiated by light. Each isomer forms its distinct primary photoproduct [3,4]. In the case of *trans* BR, this photoproduct returns via dark reactions through a series of transients to its initial isomer, trans BR (*trans* BR cycle). In the case of 13-*cis* BR, the dark pathway is split: most of the molecules return to their initial isomer, 13-*cis* BR (13-*cis* BR cycle), but some, on an alternative path, go to *trans* BR.

We report here that both cycles are connected by light-reaction pathways [5], which are occupied by photo-excitation of transients. The environmental light conditions determine the isomeric composition of BR

2. Materials and methods

Bacterioopsin (BO) was prepared by irradiating BR in the presence of hydroxylamine [6]. This BO contains retinaloxime and can be photo-reconverted to BR (unpublished results). We therefore used light at wavelengths >500 nm to irradiate samples regenerated from BO (except for the samples subjected to 'strong' light).

13-*cis* and all-*trans* retinal were purified by high pressure liquid chromatography. Regeneration of *trans* and 13-*cis* BR was accomplished by adding ethanolic solutions of the respective retinal isomers to the aqueous, buffered (pH 7) suspension of BO [3]. The ethanol concentration of the regenerated sample was $<1\%$.

By 'moderate' light we mean visible light in the wide intensity range of 10 $\mu W/cm^2$ – 500 mW/cm^2, which corresponds to the light conditions of misty to sunny days. We used a halogen tungsten lamp (150 W electric power) for the 'moderate' light (continuous light). By 'strong' light we mean visible light of intensities sufficiently large to allow absorption of 2–2000 photons/molecule within 2 ms. The latter value of 2000 photons is equivalent to an intensity of ~6000 W/cm^2 of visible light. We obtained these high light intensities by means of a xenon flash lamp (EG and G, type FX 47 C-6.5, water-cooled, 4000 J electric energy). For excitation the light of the xenon lamp was differently weakened by neutral density filters. The ultraviolet light was excluded by cut-off filters. The resulting flash is called the 'actinic flash'. Light intensities were measured photometrically and calorimetrically.

To determine the ratio of the 13-*cis* and *trans* BR isomers, we developed an analytic method based on differences in their photochemistry. The absorption change after an 'analytic' flash was measured in the ms range at room temperature at a wavelength

(545 nm), where no changes of the 13-*cis* BR cycle can be seen. 545 nm is close to the isosbestic point between ^{610}C and 13-*cis* BR. Consequently, the absorption decrease after an analytic flash represents mainly the disappearance of *trans* BR and is a direct measure of the relative amount of *trans* BR in a sample. (The 'analytic' flashes were so weak that the isomeric composition was not significantly changed.) In our experiments we subjected a sample to a single analytic flash, then light-adapted the sample with continuous 'moderate' light to transfer the 13-*cis* BR to *trans* BR, and finally subjected the sample to a second analytic flash. The ratio of the absorption changes immediately after the first and second analytic flash is the fraction of *trans* BR in the original sample. Additional details of this method are given in [3].

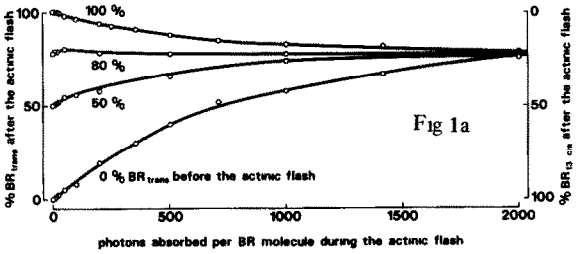
3. Results and discussion

After exposure to varying light conditions, the isomeric ratio was determined for isolated PM, regenerated *trans* BR, regenerated 13-*cis* BR, each suspended in an aqueous 0.025 M phosphate buffer (pH 6.9). We also performed experiments on living bacteria in their culture medium.

If samples containing BR were kept in the dark until no further absorption changes were detected ('dark-adapted state'), an equilibrium state of about 50% *trans* BR and 50% 13-*cis* BR was reached. The flash-analytic method is sensitive enough to detect the small temperature dependence of the equilibrium (50% *trans* BR at 0°C, 53% at 60°C) [3,4].

If BR in the dark-adapted state is now exposed to increasing light intensities, more and more *trans* BR is formed. 'Moderate' light intensity (daylight) is sufficient to transfer any of the above-mentioned samples within a few minutes or faster to a steady state of practically 100% *trans* BR [3,4]. The quantum yield of this photoreaction from the 13-*cis* BR cycle to *trans* BR is relatively low, most molecules remaining within the 13-*cis* BR cycle [3]. No photo-conversion from the *trans* BR cycle to 13-*cis* BR was detectable under these 'moderate' light intensities.

Figure 1 shows the isomeric composition of BR after it had been exposed to 'strong' light. The maximum value of 2000 photons, for example, means that each BR molecule, either as *trans* or 13-*cis* BR, or as a transient of one of the two photocycles, absorbs, on the average, 2000 photons during the length of the actinic flash. Four sets of samples, each set with a different original (pre-flash) isomeric composition, were exposed to actinic flashes of different intensity at 20°C. The original samples contained either 100%, 80%, 50% or 0% *trans* BR. Each point shown in fig.1



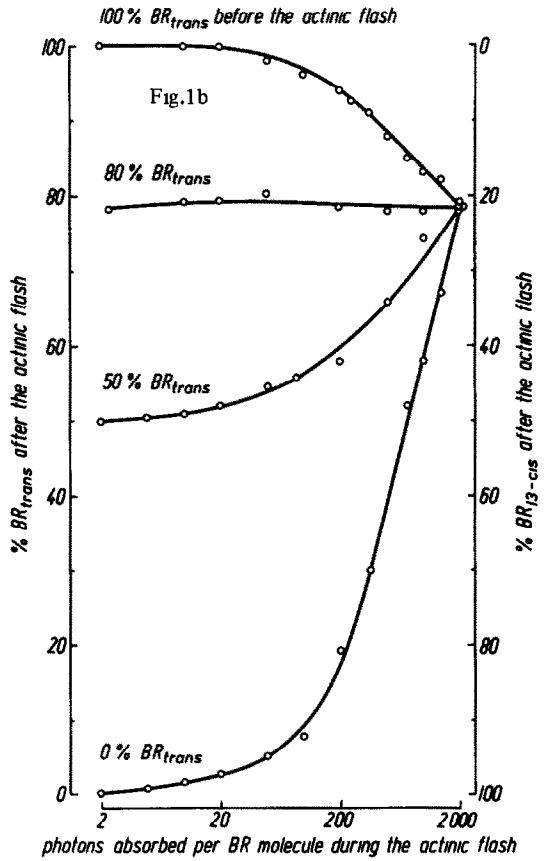


Fig 1 Isomeric composition of BR after exposure to light at different intensities Temperature 20°C (a) Linear scale, (b) abscissa logarithmic scale

was obtained using a fresh sample. Several features of the four curves should be noted: the uppermost curve (100% *trans* BR) shows that no 13-*cis* BR is formed when fewer than 20 photons are absorbed (fig.1b). At light intensities above this threshold an increasing amount of 13-*cis* BR up to the limit of about 20% 13-*cis* BR is found. In contrast, all samples containing 13-*cis* BR before the flash, show increasing amounts of *trans* BR already formed at low light intensities. No threshold was detected. The curve representing 80% original *trans* BR, for example, shows a slight increase in the beginning, then turns down and levels off to reach the final value of 80% *trans* BR at the highest intensity. Independently of their original isomeric composition, all samples of BR approach the same value of 20% 13-*cis* BR and 80% *trans* BR at the highest light intensity (fig.1a). We took great care in measuring the effect of the weak actinic flashes on the isomeric ratio (the range of up to 50 photons absorbed, fig.1b). Therefore we consider that the different behavior of 13-*cis* BR and *trans* BR in this range is well-established.

We would like to mention that the pattern shown in fig.1 holds only for the special type of flash we used in these experiments. Another type of flash (with different duration, shape, 'temperature' of the flash lamp) will yield a different pattern with a different final isomeric composition of BR. Table 1 summarizes the proportions of BR isomers under the different environmental light conditions selected.

We interpret the experimental results as follows (fig.2). BR acts as an enzyme to catalyze isomerization of the chromophore retinal at the 13-*cis* double bond. In the dark, the same thermodynamic equilibrium of the two BR isomers is always obtained in

Table 1
Isomeric ratio of BR for different light conditions at room temperature

Light conditions	*trans* BR (%)	13-*cis* BR (%)
Darkness (dark-adapted)	50	50
Moderate, continuous light e.g., 1 mW/cm² (photosteady state)	100	0
Strong light, 6000 W/cm² (2000 hν absorbed/molecule within 2 ms)	80	20

aqueous solution [3,4]. With light, each of the two BR isomers undergoes a distinct photocycle [3,4]. The two cycles differ in that there is no dark reaction from any transient of the *trans* BR cycle to 13-*cis* BR, whereas there is a dark reaction from a transient of the 13-*cis* BR cycle to *trans* BR. This transient was proposed to be ^{610}C [3]. Two dark reactions originate from ^{610}C, one leading to *trans* BR, the other to 13-*cis* BR. Because the reaction leading to *trans* BR has a much lower yield, only a small percentage of *trans* BR is formed at low flash intensities (fig.1, 0% *trans* BR curve). 'Moderate', continuous light consists of a much higher number of photons if applied over periods which are long compared to the duration of the weak actinic flash. Therefore, within a few seconds, BR is converted nearly completely to the *trans* form. Maximum *trans* BR is produced when the light intensity is strong enough to overcome the enzymatic back reaction from *trans* BR to 13-*cis* BR, and is not so strong to permit the photo-conversion from *trans* BR to 13-*cis* BR. Dark equilibration between *trans* and 13-*cis* BR occurs on the order of hours at room temperature. Photoconversion from *trans* BR to 13-*cis* BR is inferred from curves such as shown in fig.1 The fact that the 13-*cis* BR formation from *trans* BR has a threshold and is not linear with the number of absorbed photons (see upper curve, fig.1b), is explicable with the assumption of a multiple-, probably two-photon reaction. In a two-photon reaction, the first photon would start the *trans* BR cycle and the second photon would react with one of the transients to yield 13-*cis* BR. The most likely candidate is ^{630}T (also called K in the literature [7]), because at room temperature the lifetimes of the other transients are too long to account for the threshold. Assuming a reasonable quantum yield for the photoreaction ^{630}T→13-*cis* BR, the threshold can be understood quantitively. If the photoproduct, ^{630}T, obtained at room temperature, is identical with the one obtained at low temperature, our findings imply that ^{630}T undergoes different photoreactions at different temperatures At room temperature it reacts to 13-*cis* BR, at low temperatures [7] (for example liquid nitrogen temperature) it photoreacts back to *trans* BR

From an analysis of the reaction pattern of fig.1 we also propose the presence of a photoreaction from the 13-*cis* BR cycle to *trans* BR Two intermediates of the 13-*cis* BR cycle, ^{x}C and ^{610}C, cannot serve as

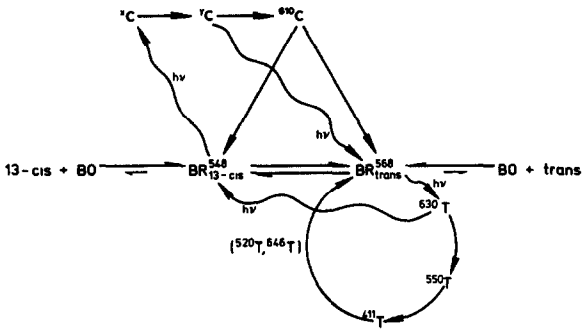


Fig.2 Simplified reaction scheme of the photochemistry and of the dark reactions of 13-*cis* and *trans* BR Products originating from $BR^{548}_{13\text{-}cis}$ after absorption of light at moderate intensity are designated as C, products originating from BR^{568}_{trans} are called T. A superscript on the right indicates the wavelength maximum of the absorption spectrum, a superscript on the left indicates the wavelength maximum of the difference absorption spectrum (spectrum of C product minus spectrum of 13-*cis* BR, spectrum of T product minus spectrum of *trans* BR). Superscripts *x* and *y* denote that the exact maxima of the difference spectra are presently unknown The notation C and T does not say anything about the configuration of the chromophore of the transients (the reader is invited to compete for the correct subscript on the right).

candidates for this photoreaction because their kinetics are either too fast or too slow. Therefore we recently introduced a new intermediate, ^{y}C [8]. New flash spectroscopic measurements in a glycerol/water mixture (2/1, w/w) confirm the existence of ^{y}C. The reaction $^{y}C \rightarrow {}^{610}C$ has a half lifetime of about 400 μs at 240 K. ^{y}C is also a red-absorbing transient, which, at 615 nm, has a higher absorption than ^{610}C.

The fact that the two BR cycles are mutually connected by photoreactions (fig.2), requires the existence of a 'photosteady state' at sufficiently high light intensities. The convergence of the four curves shown in fig.1a implies that a photosteady state is attained during the flash at the highest light intensity we applied. In the strictest sense, a photosteady state would be formed with rectangularly shaped flashes. In our case, the photosteady state will change according to the varying light intensity during the flash. The isomeric ratio of this photosteady state is not the ratio we measured after the flash, because it is altered by the tail of the flash and by the dark reactions occurring within the cycles after the flash.

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References

[1] Oesterhelt, D. and Stoeckenius, W (1971) Nature New Biol. 233, 149
[2] Oesterhelt, D , Meentzen, M and Schuhmann, L (1973) Eur. J Biochem 40, 453
[3] Dencher, N A., Rafferty, C. N and Sperling, W (1976) Berichte der Kernforschungsanlage Julich, Jul-1374, pp. 1–42
[4] Sperling, W., Carl, P., Rafferty, C N. and Dencher, N. A. (1977) Biophys. Struct Mech 3, 79.
[5] Dencher, N. A., Kohl, K.-D , Rafferty, C N and Sperling, W. (1978) Biophys J 21, 73a.
[6] Oesterhelt, D. and Schuhmann, L (1974) FEBS Lett 44, 262.
[7] Lozier, R H , Bogomolni, R A and Stoeckenius, W (1975) Biophys. J. 15, 955
[8] Sperling, W , Rafferty, C N , Kohl, K.-D and Dencher, N. A. (1978) in. Energetics and Structure of Halophilic Microorganisms (Caplan S. R and Ginzburg, M eds) pp 323–330, Elsevier/North-Holland Biomedical Press, Amsterdam, New York