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Bleaching of bacteriorhodopsin by continuous light

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Abstract A new two step photobleaching process is observed under continuous illumination of bacteriorhodopsin. This photobleaching is considerable even at physiological temperatures and becomes large at 50–60°C. The photobleaching also increases with increasing pH from 7 to 10. We suggest that the bleaching at its final stage could be due to the dissociation of the retinal and a local thermal denaturation-like process. These facts may question the generally held belief that BR is a stable protein in vivo for a long period of time. Our results may have relevance also to practical applications of bacteriorhodopsin where the stability of bacteriorhodopsin is a key issue. In certain instances, the use of bacteriorhodopsin may require cooled conditions. Here, we defined the conditions under which bacteriorhodopsin is stable. The permanent photobleaching offers a new way of picture imaging and information input for bacteriorhodopsin-based optical devices.

Key words: Retinal dissociation; Stability; Technical application; Thermal aspect of light absorption; Thermal denaturation

1. Introduction

The light driven proton pump bacteriorhodopsin (BR) functions in the purple membrane and is generally thought to be a very stable molecule. Even after standard preparation, it preserves its natural color and functions for long periods of time, possibly for years [1]. It is believed that light, even of a high intensity, does not lead to the loss of the original pigmentation of BR. However, few studies have been devoted to examining these beliefs. Only in the case of very intense laser pulses, observations were made that BR may exhibit some bleaching [2]. The temporal intensities of these laser flashes were in the MW/cm^2 range and their duration in the range of nanoseconds or shorter.

The temperature tolerance of BR is also very high. It was found to be stable up to 70–80°C [3]. Thus, on the basis of the available knowledge, there is not any reason to believe that continuous light with an intensity of a few 100 mW/cm^2 would cause any bleaching in a thermostated BR sample. The present study demonstrates that this assumption does not hold true.

We report here a new, two step photochemical reaction of BR (probably with a low quantum efficiency). Under intense light illumination, in the first step, a reversible transition and, in the second step, an irreversible denaturation-like process takes place during which the retinal is probably dissociated or released.

BR is a candidate for optoelectrical and data storage applications (see e.g. [4]). Our results may be relevant in the determination of optimal working conditions and lifetimes of these devices. The photobleaching of BR with continuous illumination may offer an alternative way of storing information in optical devices based on BR.

2. Materials and methods

The purple membranes were prepared according to standard procedures. The samples were incorporated into a 10% polyacrylamide gel and incubated at the adjusted pH in 30 mM universal buffer [5]. The experiments were done in 2 mm thick cuvettes, thermostated in a temperature-controlled brass sample holder.

The light source was a high pressure mercury lamp (200 W, HBO 200, NARVA, Berlin, Germany), filtered through heat filters and glass optical filters. The glass optical filters were transparent in the yellow spectral range ($\lambda > 500$ nm). The maximum intensity of the yellow light was approximately 400 mW/cm^2. It was measured with an Alphametrics DC 1010 photometer (Karl Lamber, Chicago, IL, USA).

The absorption spectra were measured at room temperature by a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), 1 min after switching off the illumination.

The temperature of heat denaturation of BR was measured by the phase transition-like disappearance of the absorption of the sample in the spectral range of $\lambda > 500$ nm.

3. Results

The absorption spectrum of BR changes little in the pH range of 7–10 [6]. (The shift in the absorption maximum was less than 2 nm and the decrease in the peak amplitude was only 7%). These data are in accordance with our control measurements (data not shown).

By contrast, if the samples are illuminated by an intense yellow light for a longer period (30 min), extensive bleaching can be observed in the main absorption band, as shown in Fig. 1a. The degree of bleaching depends on the pH. The 30 min of illumination resulted in an approximately 10% decrease in the absorption at pH 7, while at pH 10, the same illumination resulted in approximately 70% bleaching. Note that parallel to the bleaching, the build up of some photoproduct populations can be seen in the 320–440 nm spectral range. The characterization of these photoproducts is out of the scope of the present study. For the present, we assume that these photoproduct state(s) have deprotonated Schiff-base or, rather, they correspond to (more or less) released or dissociated retinal molecules.

The absorptions at 570 nm of the samples after the illumination are shown as function of the pH in Fig. 2a. The bleaching of BR, due to the illumination, increases linearly with increasing pH. This result suggests that titration of different BR forms or the bleaching of a special BR photocycle

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Abbreviations: BR, bacteriorhodopsin

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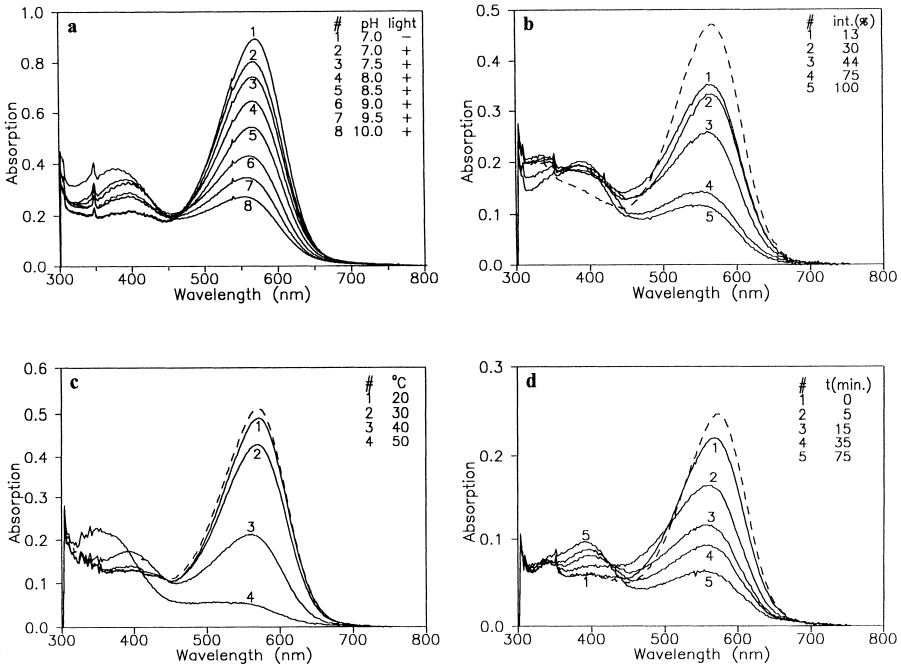

Fig. 1. Absorption spectra of BR before and after bleaching by continuous light (a) at different pH values (50°C, 30 min of illumination, circa 400 mW/cm^2), (b) at different light intensities (50°C, pH 9.5, 30 min of illumination), (c) at different temperatures (pH 9.5, 60 min of illumination, circa 400 mW/cm^2) and (d) at different bleaching times (pH 9.5, 50°C, circa 400 mW/cm^2).

intermediate are unlikely to be the origin of the bleaching phenomenon.

The dependence of the bleaching on light intensity (Fig. 1b) suggests a single photon reaction, since it fits a single exponential function, as shown in Fig. 2b.

The temperature-dependence of bleaching is very steep as it is shown in Fig. 1c. Up to 30°C, the bleaching is minimal, but at 40°C, almost half and at 50°C, practically all of the molecules are bleached. The peak amplitudes are shown in Fig. 2c. This dependence is very similar to the temperature-dependence of the thermal denaturation of BR [3], but the latter appears (in accordance to our control measurements as well) at around 80–90°C (at a high pH) [3]. This similarity suggests that the most probable mechanism for bleaching of BR by yellow light is due to some kind of a thermal denaturation process.

However, control measurements (at 40°C) in which the same effective illumination times (30 min) were applied with 20 s dark intervals followed every 10 s of illumination, proved that the sample temperature did not increase much. Although the light-induced temperature change should decrease approximately to 1/3 in this control measurement, the bleaching of the sample changed only little. Using Fig. 2c, the decrease in bleaching corresponded to only approximately a 2°C decrease in the temperature, thus, the temperature difference between the continuously-illuminated and not illuminated samples was about 3°C.

As reasonable model, it can be suggested that after light absorption, the energy released (i.e. not stored in free energy of the retinal and its surrounding) starts to spread in the protein and this local and fast decaying warming is responsible for bleaching. In such a bleaching model, one has a small localized heat source, the retinal. The heat is transferred as vibrational and rotational energy from group to group, rapidly. It seems to be plausible to assume that in the surroundings of the retinal, there exist a group, which, if it exceeds a critical temperature, results with some probability in bleaching of the BR molecule.

From the fact that the thermally-induced optically observ-

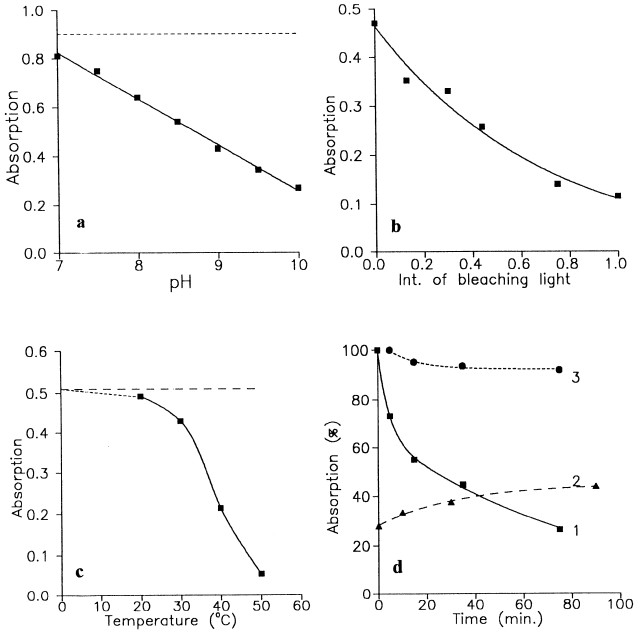

Fig. 2. The absorption maxima of the samples (at 570 nm) in the measurements shown in Fig. 1, respectively. Dashed lines in a and c show the original absorption of the samples. The solid line in b shows the best fit with one exponential function. In D, line 1 shows the bleaching versus time fitted with two exponential components (35 ± 3%; 4.4 ± 0.8 min, 65 ± 3%; 85 ± 7 min), line 2 shows some recovery of the absorption after switching off the illumination (baseline: 45%, 16.7 ± 1.3%; 34 ± 7 min) and line 3 shows the absorption of the sample stored at the measuring temperature (50°C)

able denaturation appears at around 80°C (±3°C, our result), this temperature could be taken as the critical temperature for that unknown group. The equivalent local warming after light absorption for that hypothetical group should be around 40°C. This is the temperature difference between the 'denaturation' of BR without and in the presence of 'strong' continuous light illumination.

Assuming that on the basis of the data by Makhatadze [13] a specific heat of about 1.5 J/(g K) is a fairly good value for most of the proteins. We can estimate the mass in which the energy of a single $\lambda = 500$ nm ($E = 4.0 \times 10^{-19}$ J) photon (except 20% that is stored in free energy [12]) is enough for generating this 40°C temperature difference. This mass is circa 5.3×10^{-21} g. Because one BR molecule weighs 4.3×10^{-20} g, this is about 12% of the molecule. By assuming (1) linear proportionality between the mass and volume, (2) 4.5 nm thickness of the purple membranes, (3) a 6.3 nm period of the crystal lattice, (4) a 75% BR content of the purple membrane and (5) a 2/3 fraction of the polypeptide chain inside the membrane (see e.g. [1]), the volume corresponding to this circa 12% mass fraction is about 14 nm^3. These mass and volume that can be warmed by 40°C due to a single photon absorbed corresponds to about 30 amino acids or the volume of a cube with 2.4 nm sides or a sphere with 3.0 nm diameter. Thus, from this rough estimation, one may expect that the heat sensitive group can be as far as 1.5 nm from the retinal.

Note that the denaturating group (or protein part) may be closer to the retinal than this distance. In this case, according to our model, its critical temperature should be higher than 80°C.

As interpretation consistent with our model, the pH-dependence of the photobleaching could be due to a pH-dependence of the thermal denaturation temperature of BR. In this case, the critical temperature for the thermal denaturation should decrease with an increasing pH to such an extent that accounts for the increased bleaching. The necessary change in the critical temperature for the thermal denaturation corresponding to the change in the extent of the photobleaching is shown in Fig. 2a and can be read from Fig. 2c. (Here, we assumed that the light-induced local warming is independent of the sample temperature in a limited temperature range, thus, a lower temperature of the sample would be equivalent to a higher critical temperature of the thermal denaturation in respect to photobleaching). The expected change was approximately 15°C. This was in accordance to our control measurements, in which we observed 97°C and 80°C thermal denaturation temperatures at pH 7 and 10, respectively.

Note that similar tendencies were observed by Lazarev and Shnirov [3] in the temperatures of the colorimetrically-detected reversible and irreversible denaturation components and of the spectral changes corresponding to the reversible component of the denaturation.

In this way, on one hand, we got a reasonable explanation for the pH-dependence of the photobleaching and on the other hand, our model became confirmed by other data too.

The time-dependence of bleaching due to illumination is shown in Fig. 1d and 2d (solid line). Surprisingly, it is not a monoexponential function like the light intensity-dependence of the bleaching but the sum of two exponential components. This apparent contradiction between the two data sets suggests a model for bleaching. This is that $A \leftrightarrow B \rightarrow C$, where A is the light sensitive state, probably the BR ground state, B is the primary photoproduct which is in equilibrium with A under illumination (due to thermal transition from B to A) and C is the secondary, irreversible product of the bleaching.

From the mathematical analysis of the data, it is obvious that first the $A \leftrightarrow B$ equilibrium is set by light and due to this, the bleaching of A is more rapid because this reaction is one order of magnitude faster than the reaction from B to C.

Singular value decomposition (SVD) analysis of the data in Fig. 2d shows three significant spectral components, with characteristic maxima of 570, 490 and 390 nm, respectively. These findings provide strong support for the model proposed above.

The model predicts that after switching off the light, some repopulation of A (i.e. BR) should occur. This is confirmed experimentally by the data shown in Fig. 2d, line 2 (triangles).

As a further control experiment, we checked if BR becomes bleached during storage at the measuring temperature (50°C). Although some decrease in the absorption can be seen (Fig. 2d, line 3, circles), the effect is negligible compared to the extent of bleaching caused by light.

The extent of bleaching of BR by light was not influenced by the addition of 2 M NaCl.

4. Discussion

A novel phenomenon, the bleaching of BR by continuous yellow light, is demonstrated in this paper. The intensity of this light is similar to ambient daylight. The highest intensity was only 2–5 times higher than that was measured in a sunny day in our country [7]. BR is one of the best candidates as a biological material for data storage [4]. Our results offer a new effective way to this, but these data also point out that the stability of devices using reversible photoprocesses may be limited by such bleaching phenomena, especially at higher temperatures (approximately above 35°C).

The photodestruction of BR by laser flashes reported by Czégé and Reinish [2] is probably a different process from the bleaching by continuous light reported here. In our case, no red-shift of the remaining BR occurs, the product of the bleaching apparently has a higher wavelength of absorption maxima (circa 390–400 nm) and the process is independent from the addition of 2 M NaCl.

It should be noted that in solubilized halorhodopsin, a continuous light caused bleaching phenomena reported by Zimányi and Lanyi [8]. They found that the product of bleaching is a dissociated 9-*cis* chromophore. According to their, and our, absorption spectra, it is likely that in the case of BR bleaching, the dissociated retinal chromophore is also one of the photoproducts.

We suggest a theoretical model for the bleaching of BR by continuous illumination. This model and the results of our SVD analysis are in good agreement with the temperature denaturation data, where also three similar components were found [3]. Although this model is somewhat hypothetical, it provides a good description of the observed phenomena and directs attention to the thermal aspect of light absorption in proteins. The majority of the light energy absorbed is dissipated almost immediately through the protein, causing considerable warming in a relatively large volume of it.

Note that in some respect, a similar thermal effect is suggested to be one of the possible causes of the light-induced

structural changes in macroaggregates of light harvesting chlorophyll pigment-protein complexes [9–11].

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