THE PHOTOCHEMICAL REACTION OF THE 412 nm CHROMOPHORE OF BACTERIORHODOPSIN

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1. Introduction

The purple membrane of *Halobacterium halobium* converts light energy into electrochemical energy [1,2]. Light absorbed by Bacteriorhodopsin (BR 570) induces a reaction cycle which consists of two main parts: First, BR 570 is transformed to a relatively stable product absorbing at 412 nm (BR 412) under proton release into the medium, in the second part of the cycle BR 570 is regenerated under proton uptake [2–5]. While in the first reaction sequence several short-life intermediates could be identified [4,6–8], the mechanism of the regeneration process is not understood. Some time ago, we observed the acceleration of the proton uptake of bleached Bacteriorhodopsin by illumination with blue light [2,9], an interaction which proved to be useful for the study of the regeneration process. Here, we report that this light activated regeneration of Bacteriorhodopsin BR 570 fulfills the criteria of a photochemical reaction and that the regeneration kinetics can be described by two exponentials, which involve two different forms of the BR 412 chromophore, as well as a component in addition to the BR 570 chromophore, preliminary results of which have been reported elsewhere [9,10].

2. Material and methods

Growth of *Halobacterium halobium* (mutant NRL R_1M_1), isolation of the purple membrane as well as the incubation techniques were those described earlier [2,11]. In addition experiments were also carried out in purple membrane suspended in a water/ethyleneglycole mixture (1:1 = v/v).

Spectrophotometric measurements were carried out in a spectrophotometer [2] using the double beam or single beam mode, respectively and occasionally filters of Schott, Mainz, were used for light selection. Rapid scanning and purple membrane bleaching techniques were those described elsewhere [2]. An actinic light system (400 W Xenon arc, Bausch and Lomb monochromator No. 33-86-02) was arranged rectangular to the photometric system. The relative actinic light intensity was recorded via a splitted fraction of the primary light exciting a Rhodamin B fluorescence. The fluorescence output was calibrated in terms of absolute light intensity (Einsteins $\times$ min^{-1}) with a chemical Ferrioxalate actinometer [12]. In addition the light intensity was measured using the rate of its photochemical decomposition of carbonmonoxide myoglobin as intensity indicator [13]. The temperature was controlled with an Air Liquide equipment and recorded with $\pm$ 1%°C. In all experiments an overlap up to 10 s between the bleaching phase and the actinic light activation phase was maintained to obtain a photochemical pre-equilibrium. The intensity of the measuring light was small enough not to photoactivate the BR 570 component.

Kinetic analyses were carried out with semilogarithmic plots of the reaction progress curves of the BR 570 regeneration after its complete bleaching in the dark and under illumination with light between 380–460 nm. The biphasicity was analyzed by resolving the two first-order exponentials graphically. While constants for the slower partial reaction were calculated from half-times, the constants for the faster reaction

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were usually computed from initial reaction velocities according to:

$$k_I = \frac{k'}{A_1} - k_{II}\frac{A_2}{A_1}$$

where A_1 and A_2 are the amplitude fractions of reaction I and II respectively constituting the normalized BR 570 regeneration kinetic, k' is the constant obtained from initial velocity and k_{II} the constant of the partial reaction II.

Quantum yields (ϕ) were calculated from the difference between the first-order constants obtained in the dark and in the presence of actinic light (412 nm) according to:

$$\phi = \frac{\Delta k\ [PM]\ AF\ V}{I_0\ (1-T)}$$

where $\Delta k = k$ (light at 412 nm) $- k$ (dark), AF = amplitude fraction of reaction I and II resp., [PM] = purple membrane concentration (M^{-1}), V = volume, I_0 = light intensity (Einsteins × min^{-1}) and T = transmission. The absorbed quanta were obtained with the absorption coefficient $\epsilon = 33\ mM^{-1} \times cm^{-1}$ for BR 412 [2].

3. Results

3.1. *Dark and light activated BR 570 regeneration*

The regeneration process of BR 570 from its fully bleached state in the dark can be time resolved at lower temperature as two first-order processes running parallel in first approximation (fig.1A). The first part of the reaction, reaction I (see also insert) constitutes 17% of the total amplitude being approximately 30× faster (k_I=6.7 min^{-1}) than reaction II (k_{II}=0.23 min^{-1}) the latter constituting 83% of total. The average contribution of each reaction is 20% for the first and 80% for the second reaction.

The influence of actinic light at 412 nm is demonstrated in fig.1B. The light enhanced velocities yielded constants k_I=12.6 min^{-1}, k_{II}=0.82 min^{-1} and a relative decrease of the amplitude of reaction I to about 13%. This decrease is due to the technique of experimentation where the actinic light of 412 nm was turned on a few seconds before the bleaching light was turned off, and a constant photochemical pre-equilibrium was reached. Consistent with a decrease of the amplitude

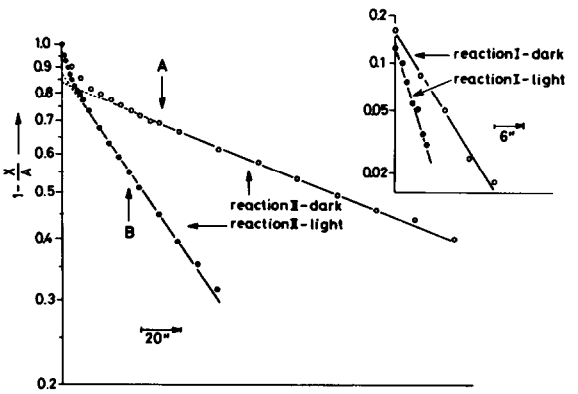


Fig.1. Semilogarithmic plots of the reaction progress curves of the regeneration of the completely bleached purple complex in the dark (A) and in presence of light (412 nm) (B) measuring light 576 nm, purple membrane (3 × 10^{-5} M) −12°C.

of reaction I is a decreased amplitude of the recovered BR 570 as shown below.

The biphasic BR 570 regeneration and the light activation of both reactions is also observed in other medium conditions, such as a water–ethyleneglycol mixture at −40°C.

3.2. *Quantum efficiency*

Figure 2 shows that the light enhancement of the two partial regeneration velocities is a function of the light intensity. It is interesting to note that saturation of reaction I occurs at lower light intensity than reaction II. From the initial linear part of the plots obtain-

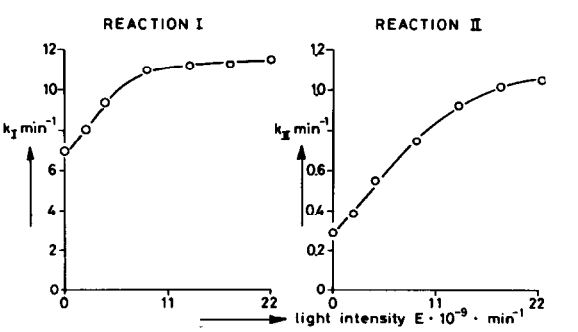


Fig.2. Dependency of the first-order rate constants of the partial reaction I and II of the BR 570 regeneration on actinic light intensity (Einsteins × min^{-1}) purple membrane (10^{-5} M, −12°C).

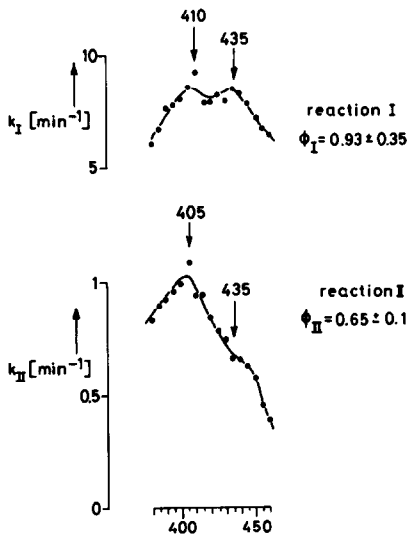


Fig.3. Action spectra for the reaction I and II of light activated BR 570 regeneration.

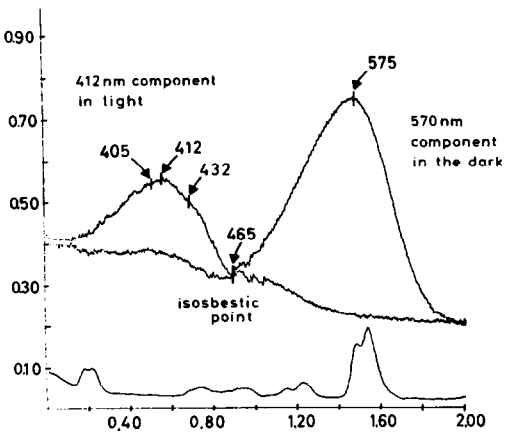


Fig.4. Absorption spectra of BR 570 and BR 412. The spectra were obtained after repetitive laser flash activation up to saturation, purple membrane (1.5×10^{-5} M) plus 25% (v/v) ethyleneglycol −30°C. The lower spectrum represents the neodymium filter for calibration. Wavelength scale is given in arbitrary units. Ordinate is given in relative extinction units.

ed with different purple membrane concentrations ($1-4 \times 10^{-5}$ M) at temperatures between −6°C and −16°C, quantum yields of ϕ_I=0.93 ± 0.35 and ϕ_{II}= 0.65 ± 0.1 (n = 8) for reaction I and II respectively were obtained.

3.3. *Action spectra and absorption spectrum of BR 412*

When the wavelength of the actinic light at constant intensity is varied from 380–460 nm and the first-order constants of the two partial reactions of BR 570 regeneration rate are plotted against the wavelength. The action spectra for both reactions are obtained as shown in fig.3, which clearly indicates two different action spectra for both reactions. Reaction I is characterized by two maxima of 410 nm and 435 nm, reaction II with a new maximum at 405 nm and a shoulder at 435 nm.

The composed structure of the action spectra for reaction I and reaction II let us to re-examine under similar conditions the absorption spectrum of BR 412 using a repetitive laser flash saturation for complete bleaching. As shown in fig.4 a highly inhomogeneous spectrum is obtained after bleaching with a clear shoulder observed at 432 nm, which is hardly seen in the absorption spectrum of BR 412, when BR 570 is incompletely bleached (see [2]). Action spectrum and absorption spectrum clearly indicate a composite structure of the BR 412 chromophore. It is concluded that this spectrum consists of two overlapping components similar but not identical.

3.4. *Regeneration product*

In order to test the structure of the regeneration product, a spectral amplitude analysis after complete

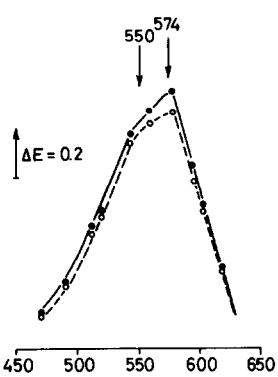


Fig.5. Recovery spectra of BR 570 obtained after complete bleaching in the dark (closed circles) and in the presence of light of 412 nm (open circles), purple membrane 2×10^{-5} M, −10°C).

regeneration as a function of the wavelength was carried out as shown in fig.5. This spectrum shows that the regeneration product with a known maximum of absorption at 570 nm is not homogeneous, but contains an additional component as indicated in the shoulder at approximately 550 nm. This component could not be kinetically resolved under the conditions used, however component analysis shows that it amounts to approximately 40% amplitude size with a maximum around 520 nm. It is interesting to note that this component does not show up in the spectrum given in fig.4 as well as fig.2 of [2].

4. Discussion and conclusions

The light activated regeneration of BR 570 of the photochemical cycle of Bacteriorhodopsin allows to define an action spectrum of the relatively stable BR 412 component as function of light intensity and wavelength and to refine the composition of BR 412 as well as the simple action spectrum reported earlier [9,14]. The kinetic differentiation yields two action spectra with different quantum yields.

The second photoreaction of the overall Bacteriorhodopsin cycle leads to a two-fold photochemical equilibrium (see also [14]) as is clearly shown in the amplitude sensitivity of fig.5, where the difference between the two light activated states is recorded for the photo-pre-equilibrium conditions used.

The two different action spectra also redefine the structural composition of the BR 412 chromophore and indicate their function within the overall photochemical cycle on the following ground:

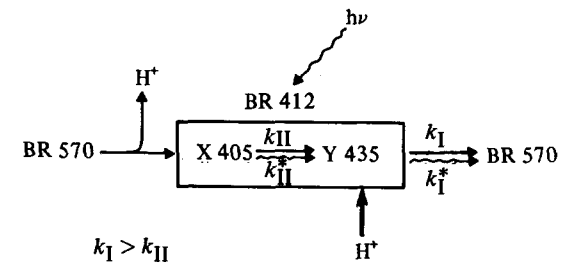
(1) Under blue light illumination the steady state equilibrium between BR 412 and BR 570 is shifted towards BR 570 on the expense of partial reaction I.

(2) The quantum yield of reaction I is nearly 50% higher than that of reaction II.

(3) The action spectrum of reaction I and II indicate two components X 405 and Y 435 a result which is also confirmed by the inhomogeneous absorption spectrum of BR 412 with a shoulder of 432 nm under conditions of fully bleached BR 570.

On the other hand the maximum around 410 nm in the action spectrum I and the shoulder at 435 nm in the action spectrum II indicate that the two components are coupled. It might well be that the two species with different polarization and different life times as observed by Slifkin and Caplan [15] are identical with the components identified here directly.

Based on our considerations, the following reaction pathway is proposed:



Under photochemical equilibrium conditions two forms of BR 412 can be observed: X 405 and Y 435, the latter being the regeneration precursor of BR 570, and reaction II being the rate-limiting process.

Our experiments also show that the regeneration kinetic is not only biphasic in the light but also in the dark. Thus, reaction II is now identified as the rate-limiting step of the overall cycle for the conditions used. We here again raise the question of the coupling mechanism of the photochemical reaction pathway and the deprotonation and protonation step occurring at two different conformations of the protein as defined by the spectral property of Bacteriorhodopsin.

So far it is known that the deprotonation of Bacteriorhodopsin correlates with the formation of BR 412 and probably X 405 [3,10]. The protonation step should be coupled to the rate of formation of BR 570. However no clear correlation with any of the components has yet been reported, although in acid and base component structure of Bacteriorhodopsin absorbing at 640 nm and 550 nm for pH 8 and pH 5 has recently been suggested [16].

Acknowledgements

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