A PHOTOSENSITIVE PRODUCT OF SODIUM BOROHYDRIDE REDUCTION OF BACTERIORHODOPSIN

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1. Introduction

Halobacterium halobium is able to convert light energy to chemical free energy [1,2]. In this process light is absorbed by the pigment bacteriorhodopsin (bR) which occurs in the bacterial cell membrane in crystalline [3] arrays. These arrays can be isolated and have been termed the purple membrane [4,5]. In the purple membrane, bacteriorhodopsin is the only protein present [6]. It contains retinal bound to a lysine residue [7] in the form of a Schiff base and closely resembles the visual pigment of animals. The visible absorption maximum of bacteriorhodopsin occurs at 570 nm. The position of this maximum is considerably red shifted as compared to simple Schiff bases of retinal and is determined by non-covalent interaction of retinal with the protein. Upon absorption of light, bacteriorhodopsin undergoes a cyclic photoreaction [8], accompanied by the translocation of protons from one side of the membrane to the other. The photoreactions of rhodopsins and bacteriorhodopsin are similar in many respects.

Neither native rhodopsin nor bacteriorhodopsin react with sodium borohydride ($NaBH_4$) in the dark. When the pigments are modified, e.g. by exposure to certain detergents, the Schiff base linkage is reduced by $NaBH_4$. The resulting retinyl proteins have absorption spectra with maxima at 330 nm and 280 nm, essentially equivalent to the sum of the spectra of the retinyl chromophore and the opsin [6,9,10]. It has been reported that bacteriorhodopsin also reacts with $NaBH_4$ in the light [11]. The authors mentioned briefly that purple membranes become discolored if illuminated in 1% unbuffered $NaBH_4$. The discolored membranes were said to show the typical retinyl protein fluorescence and did not recombine with retinal to yield bacteriorhodopsin. An absorption spectrum of the discolored membranes was not given nor a photoreaction mentioned. We find that the $NaBH_4$ reaction results in a new pigment with an absorption maximum red shifted as compared to the retinyl protein obtained after detergent treatment and reduction. Furthermore, this pigment (bR^{red}_{360}) exhibits a characteristic photoreaction. We conclude that in bacteriorhodopsin the $NaBH_4$ reaction in the light apparently modifies the covalent linkage between chromophore and protein while preserving existing or possibly establishing new non-covalent interaction(s).

2. Materials and methods

Purple membrane was isolated from *H. halobium* R_1 as described [4,5]. Samples were suspended in freshly prepared 50 mM carbonate buffer, pH 10.0, to yield a suspension of $A_{570} = 1$. Approx. 10 ml of this suspension was cooled to 0°C and mixed with 15 mg/ml powdered $NaBH_4$. The membranes were then illuminated at 0°C in a thermostated test tube of 25 mm diameter with a Sylvania Colorarc 300/16 lamp whose light was filtered through 15 cm of a 2% $CuSO_4$ solution and an orange long-wavelength pass filter (Schott OG2, 3 mm). The filter combination had its maximum of transmission at 570 nm; 50% of maximal transmission occurred at 560 nm and 590 nm. The light intensity at the position of the sample was $\sim 1-5 \times 10^5$ erg $\times$ cm^{-2} $\times$ sec^{-1} as measured with a Kettering radiant power meter (Laboratory Data Con-

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trol, Div. Milton Roy Co., Riviera Beach, Florida). Depending on the light intensity the membranes were bleached within 10 to 30 min and were then washed several times in 150 mM Na-phosphate buffer, pH 7.0, by centrifugation and resuspension until residual $NaBH_4$ was completely removed (see also below). The reaction can also be carried out in 'basal salt' (growth medium without peptone) adjusted to pH 10. This procedure yields the same product as the reaction in carbonate buffer but the reaction is complete in half the time with half the amount of $NaBH_4$. It may be noted that the reaction rate is slow in both procedures as compared to rhodopsin or bacteriorhodopsin after denaturation with detergent. Absorption spectra were recorded with a Cary 14 spectrophotometer equipped with a scattered transmission accessory. The spectrofluorimeter described by Cheung and Morales [12] was used, without side path for a quantum counter and without ratio detector. The angle between the excitation beam and the direction of observation was 90°. Fluorescence decay times were measured with the instrument described by Mendelson, Morales and Botts [13].

3. Results

In order to obtain the kinetics of the $NaBH_4$ reaction (fig.1) a suspension of purple membrane in carbonate buffer, pH 10.0, was mixed with $NaBH_4$ as described. One part of the reaction mixture was removed and kept in the dark at 0°C, the remainder was illuminated. Samples of the illuminated membrane suspension were removed after 3, 6, and 12 min respectively and kept in the dark. After the last sample had been obtained all samples were washed and resuspended in phosphate buffer, pH 7.0, to yield the same optical density at 280 nm. This procedure was chosen because foaming made it difficult to obtain spectra in the presence of $NaBH_4$. The choice of the optical density at 280 nm as a reference point may, of course, be questioned; however, the result – especially the occurrence of an isosbestic point at 405 nm approximately – indicates that the error which might be introduced in this manner is small.

Fig.1 shows that the purple membrane does not react with $NaBH_4$ in the dark but only when illuminated with light absorbed by bacteriorhodopsin. The product of the reaction in the light (bR^{red}_{360}) has an absorption maximum at 361 nm with shoulders at approximately 342 nm and 380 nm. Comparing the extinction at 361 nm with the 570 nm band of bacteriorhodopsin ($\epsilon_{570} = 63 \times 10^3\ M^{-1}\ cm^{-1}$; [14]) the extinction coefficient of bR^{red}_{360} is estimated to be $\epsilon_{361} \simeq 50 \times 10^3\ M^{-1}\ cm^{-1}$.

The structure of the $NaBH_4$-reacted purple mem-

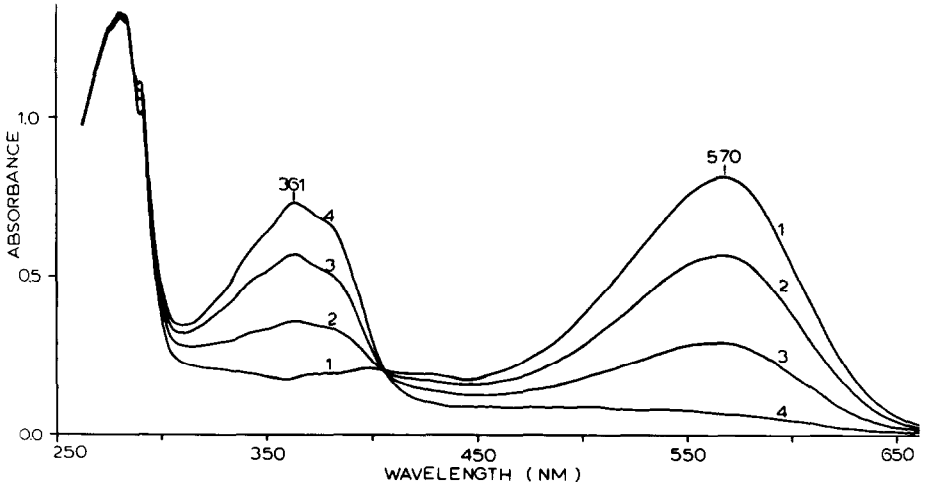


Fig.1. The reaction of the purple membrane with sodium borohydride ($NaBH_4$). A suspension of purple membrane was incubated with $NaBH_4$ as described in the text. Curve 1: membranes kept in the dark. Curves 2 to 4: membranes illuminated with light of 570 nm and 4×10^5 erg × cm^{-2} N × sec^{-1} for 3 min (curve 2), 6 min (curve 3) and 12 min (curve 4).

brane has been examined. Electron microscopy of platinum-carbon shadowed dried membranes reveals no differences between samples before and after reaction with $NaBH_4$; the X-ray diffraction pattern of the $NaBH_4$-reacted purple membrane shows the same hexagonal lattice which is found in the unmodified membrane (personal communication, G. King); the circular dichroism spectrum of the $NaBH_4$-reacted purple membrane indicates a high content of α-helical structures, which is comparable to that of the unmodified membrane (personal communication, Y.-W. Tseng).

The chromophore in bR^{red}_{360} is not extractable with organic solvents, e.g. petroleum ether (boiling range 30 to 60°C) or chloroform. In a mixture of $NaBH_4$-reacted purple membrane and all-trans-retinal in which bR^{red}_{360} and retinal were present in approximately equal molar amounts, no indication for a reconstitution of bacteriorhodopsin was observed within 90 min at room temperature. The reconstitution of bacterio-opsin (b-opsin) with all-trans-retinal is complete in 30 min [11]. These experiments indicate that the chromophore in bR^{red}_{360} is covalently and stably attached to the protein and that it is still bound at the original site.

We assume that the reaction with $NaBH_4$ reduces the Schiff base linkage between retinal and b-opsin to a C–N single bond and this is supported by the observation shown in fig.2a. After removing excess $NaBH_4$ addition of 10 mM CTAB shifts the absorption maximum of bR^{red}_{360} to 330 nm and the spectrum assumes a shape which is typical for retinyl-protein conjugates (cf. the spectrum of *N*-retinyl-opsin in [9]). These spectral changes are not due to further reduction of the chromophore after denaturation by residual $NaBH_4$. If retinal is added to the washed suspension of bR^{red}_{360} it is not reduced to retinol but can be completely recovered by extraction into hexane, although addition of CTAB still produces the shift to 330 nm (bR^{red}_{330}). Further confirmation of the reduction of the Schiff base linkage is obtained by an experiment in which the purple membrane is first solubilized with CTAB, thus forming *N*-retinylidene-b-opsin and then reduced with $NaBH_4$ to *N*-retinyl-b-opsin (fig.2b). The spectra of *N*-retinyl-b-opsin produced by these two reactions are identical.

bR^{red}_{360} undergoes a characteristic conversion upon illumination with UV light or after incubation with

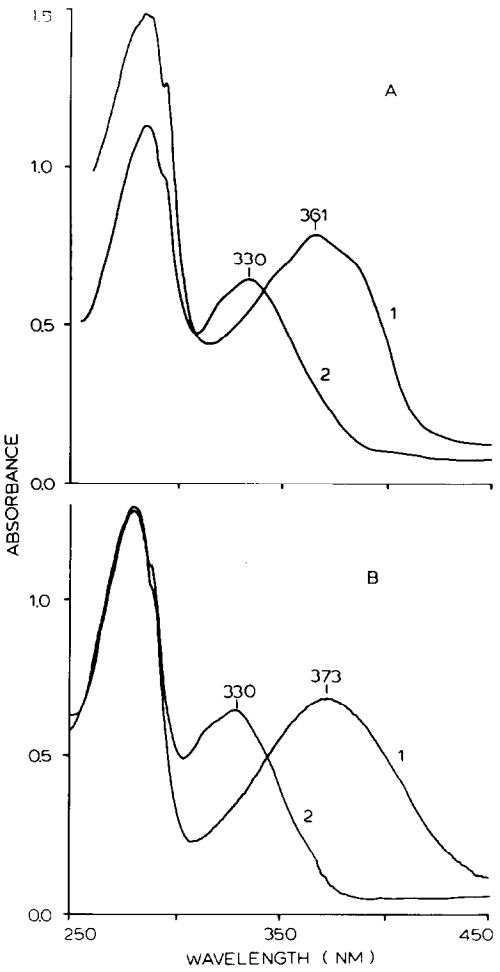


Fig.2. Evidence for the reduction of the Schiff base linkage in bacteriorhodopsin by reaction with $NaBH_4$. (a) Addition of the detergent cetyltrimethyl ammonium bromide (CTAB), final concentration 10 mM, to a suspension of $NaBH_4$-reacted purple membrane (bR^{red}_{360}), pH 7.0, (curve 1) yields a product, bR^{red}_{330} (curve 2), with a characteristic *N*-retinyl-protein spectrum. (b) The same N-retinyl-protein spectrum is obtained if a suspension of purple membrane in phosphate buffer, pH 7.0, is first treated with CTAB (curve 1) and immediately reduced with ∿ 0.5 mg/ml of $NaBH_4$ (curve 2).

concentrated hydrochloric acid. Fig.3 shows the kinetics of this transition as seen upon broad band irradiation with light of 350 nm to 400 nm at 2×10^4 erg × cm^{-2} × sec^{-1}. The absorption spectrum of the newly formed compound has a pronounced fine structure with maxima at 327, 343, 361 and

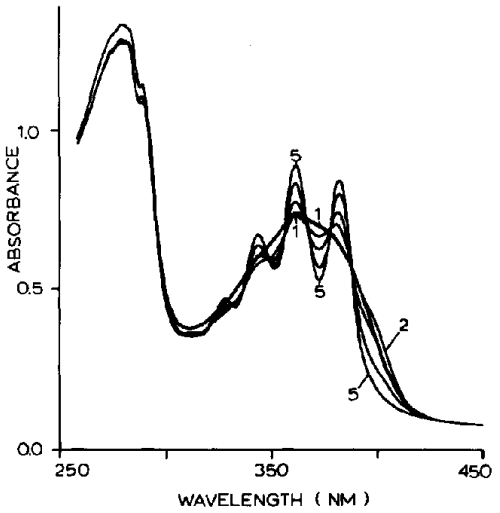


Fig.3. Kinetics of the photoreaction of bR^{red}_{360}. A suspension of $NaBH_4$-reacted purple membrane (bR^{red}_{360}), pH 7.0, is exposed to u.v. light (λ = 350–400 nm, intensity: 2 × 10^4 erg × cm^{-2} × sec^{-1}) at 0°C. Curve 1: 0 time; curve 2: after 3 min; curve 3: after 14 min; curve 4: after 34 min; curve 5: after 50 min of u.v. exposure.

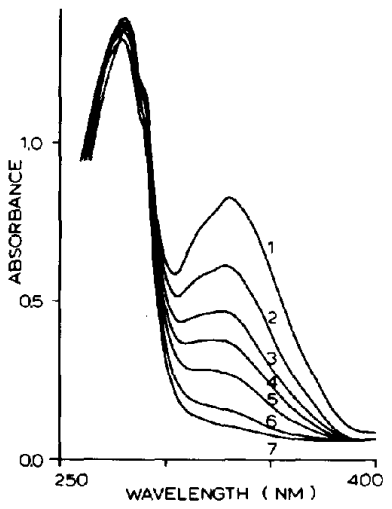


Fig.4. The photodestruction of *N*-retinyl-b-opsin. A suspension of $NaBH_4$-reacted purple membrane (bR^{red}_{360}), pH 7.0, was dissolved in 10 mM CTAB yielding bR^{red}_{330} and then exposed to the light of a 200 W high pressure mercury lamp filtered by a u.v. filter (Corning 7–54). Curve 1: 0 time; curve 2: after 10 sec; curve 3: after 20 sec; curve 4: after 38 sec; curve 5: after 68 sec; curve 6: after 148 sec; curve 7: after 300 sec of u.v. exposure.

382 nm. The kinetics show that the spectra obtained after increasing times of u.v. irradiation (curves 2–5) have isosbestic points. These isosbestic points, however, are not shared by the spectrum of the original reduced membrane suspension bR^{red}_{360} (curve 1). It thus follows that the reaction proceeds in more than one step. The same conversion has been observed upon monochromatic excitation at 365 nm or at 280 nm. Illumination of a preparation reacted with $NaBH_4$ after addition of CTAB, bR^{red}_{330}, however, merely leads to the destruction of the chromophore (fig.4) in a similar manner as reported for retinol [15]. The conversion of bR^{red}_{360} can also be produced in the dark by incubation with hydrochloric acid; its rate depends on the concentration of HCl: in 1.6 N HCl it is complete in about 24 h at room temperature; in 6.6 N HCl (fig.5) the same effect is obtained within 2 h. The kinetics of the conversion in HCl has a slightly different appearance than the kinetics of the photo-reaction (fig.3). This, however, seems to be caused by a time-dependent change of light scattering rather than by a real difference in the conversion reaction: the membrane sample in HCl was quite turbid at the beginning of the incubation but in time

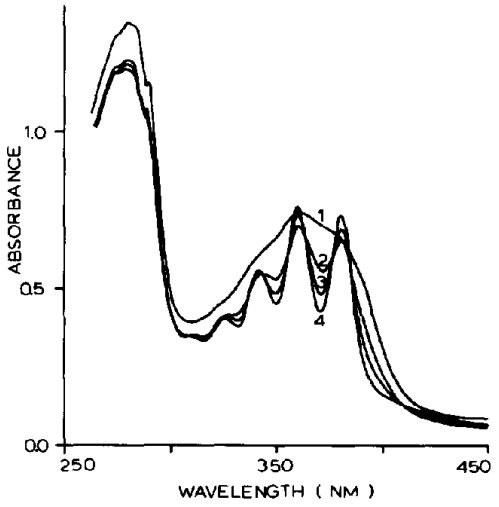


Fig.5. The effect of concentrated hydrochloric acid on bR^{red}_{360}. A suspension of $NaBH_4$-reacted purple membrane (bR^{red}_{360}), pH 7.0, was mixed with concentrated HCl (final concentration 6.6 N) in the dark. Curve 1: without HCl; curve 2: immediately after addition of acid; curve 3: 15 min later; curve 4: 120 min later.

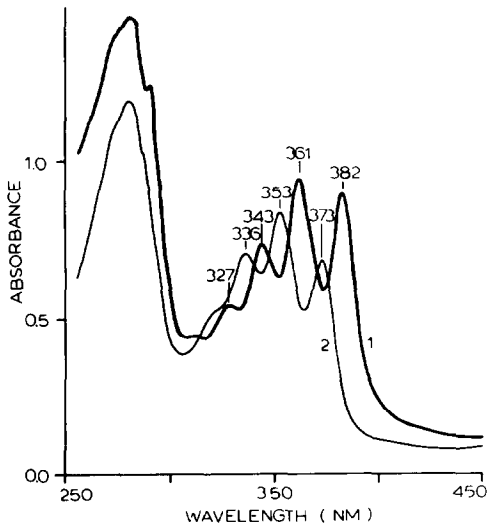


Fig.6. The effect of detergent on the photoconverted form of bR^{red}_{360}. Addition of CTAB (final concentration 10 mM) to a suspension of $NaBH_4$-reacted and photoconverted purple membrane (curve 1, $\lambda\lambda_{max}$ = 327, 343, 361 and 382 nm) causes a small shift in absorption maxima and a slight change in the shape of the spectrum (curve 2, $\lambda\lambda_{max}$ = 320 (S), 336, 353 and 373 nm).

became progressively clearer. Addition of CTAB to the converted form of bR^{red}_{360} produces a small blue shift of the absorption spectrum, and there is a slight change in the shape of the spectrum while the fine structure is retained (fig.6).

As may be expected for retinol derivatives, bR^{red}_{360} and its converted form both are highly fluorescent. The fluorescence excitation and emission spectra of the converted form of bR^{red}_{360} are given in fig.7. The excitation spectrum has maxima at the same positions as the absorption spectrum. The occurrence of a maximum in the excitation spectrum around 280 nm indicates energy transfer from aromatic amino residues of b-opsin to the chromophore and thus explains why the conversion of bR^{red}_{360} can be produced by irradiation with light of 280 nm. The emission spectrum is not a mirror image of the excitation spectrum and exhibits far less structure. A similar relation has been reported for the absorption and emission spectra of anhydroretinol [16], which like retroretinol (see below) has a 'retro' configuration. The converted form of bR^{red}_{360} has also been studied by nanosecond fluorescence spectroscopy (in collaboration with R. Mendelson). A simple exponential decay with a

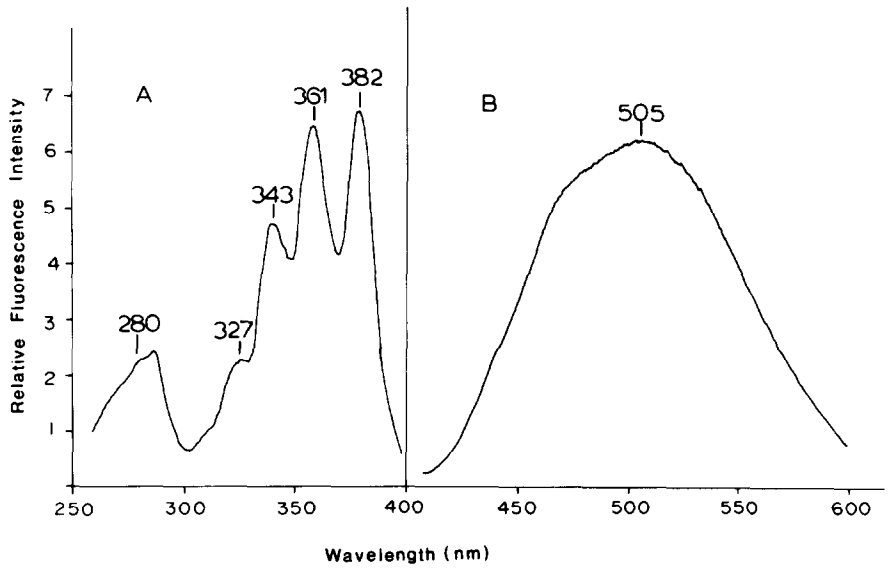


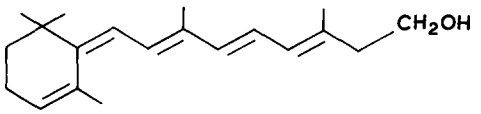
Fig.7. Uncorrected fluorescence spectra of the $NaBH_4$ reacted and photoconverted purple membrane. (a) Excitation spectrum with emission monochromator set at 505 nm. (b) Emission spectrum with excitation at 361 nm.

lifetime of τ = 20.0 nsec was found. This lifetime is long compared to retinol fluorescence [17,18]. The lifetime of retinol depends on environmental factors, in particular on the polarity of the solvent. Values ranging from 1.5 nsec for retinol in methanol to 11.0 nsec for retinol in dipalmitoyl lecithin/cholesterol (70 : 30 mol %) have been reported [18].

4. Discussion

The question arises why a pigment comparable to bR^{red}_{360} has not been observed in connection with rhodopsin although the photoreactions of both pigments are rather similar. The answer may be found in the different organization of the purple membrane and of photoreceptor membranes of higher animals. Whereas the purple membrane consists of a rigid two-dimensional crystalline array of bacteriorhodopsin molecules [3,19–22], photoreceptor membranes of higher animals are in a fluid rather than a crystalline state. Rhodopsin shows a high rate of rotational and translational diffusion in the disc membranes of rod outer segments [23–26]. It seems reasonable to assume that the crystalline structure of the purple membrane increases the stability of the protein conformation around the chromophore and thus permits the reduction of the Schiff base linkage while preserving non-covalent interaction between chromophore and protein. The possibility remains that a pigment analogous to bR^{red}_{360} appears transiently upon reaction of rhodopsin with $NaBH_4$, but because of rapid decay has not yet been detected.

In contrast to the blue-shifted and little structured spectrum of retinol [27], the spectrum of the chromophore in the photoconverted form of bR^{red}_{360} shows the three main peaks expected for polyenes with five conjugated double bonds. There is a small number of retinal derivatives whose spectra closely resemble the converted form of bR^{red}_{360} ([27,28] Rosalie Crouch, A. Kropf, personal communication); however, considering possible reactions in the photochemical cycle of bacteriorhodopsin we felt that the most likely chromophore to occur in the converted form of bR^{red}_{360} is an all-trans retroretinyl residue, a retinal derivative in which all double bonds are shifted towards the ring by one position. Retroretinol has the following structure:



In light petroleum it shows absorption maxima at 332, 348 and 367 nm [29]. The absorption spectrum of all-trans-retroretinol methyl ether in 95% ethanol has been given by Oroshnik et al. [27] and shows maxima at the same positions. This may be compared with the 336, 353 and 373 nm maxima for the converted form of bR^{red}_{360} in detergent (fig.6). The slight differences in the positions of the maxima could be due to the different solvents and a small degree of residual interaction with the protein. Retroretinol has attracted little attention in the past [27,29–36] and has not been observed in connection with visual pigments so far.

The fact that bR^{red}_{360} is formed only in the light lets us expect that it will provide important clues to the chemistry of the photoreaction cycle once its exact nature has been established. In addition to these aspects the $NaBH_4$ reaction provides a method to introduce a fluorescent group into bR and to study the function of the purple membrane by fluorescence techniques.

Acknowledgements

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