LIGHT-DEPENDENT REACTION OF BACTERIORHODOPSIN WITH HYDROXYLAMINE IN CELL SUSPENSIONS OF *HALOBACTERIUM HALOBIUM*: DEMONSTRATION OF AN APO-MEMBRANE

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Received 17 May 1974

1. Introduction

Vitamin A aldehyde occurs in *Halobacterium halobium* bound to a rhodopsin-like protein, Bacteriorhodopsin [1]. This is the only protein in the purple membrane which forms patches in the surface membrane of the cell [1,2]. Bacteriorhodopsin mediates light energy conversion via a cyclic photochemical reaction accompanied by proton release and uptake [3–6]. This reaction was studied first in detail in a salt–ether solvent system using purified purple membrane suspensions [4]. So far, two spectroscopically distinct forms of the chromophore in bacteriorhodopsin – the purple complex and the 412-nm complex – have been shown to participate in the cycle. Only the latter reacts with hydroxylamine to yield retinaloxime (ref. [7], p. 461).

We report here on the occurence of retinaloxime formation in the intact illuminated cell, if the medium contains hydroxylamine. Furthermore a first analysis of the distribution of the second reaction product bacterio-opsin within the cell membrane is given and third the preparation of a chromophore-free membrane, a white apo-membrane of the purple membrane is described.

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2. Materials and methods

H. halobium NRL R_1M_1 cells were grown as described in [7]. Cell suspensions were concentrated by centrifugation and resuspension in medium without peptone (basal salt).

Aliquots were placed into two cuvettes and 2 M NaCl solution, pH 7, with and without hydroxylamine was added. Both cuvettes were then illuminated uniformly at room temperature under stirring with light from a Xenon high pressure lamp (900 W) filtered through 25 cm water, 2 cm 5% $CuSO_4$ solution and a 4 mm orange glass filter (OG_4, Schott, Jena) and the difference spectra recorded with a Zeiss spectrophotometer DMR 21*. A spectral range from 510 to 710 nm was selected by the filter combination used. The light intensity at the position of the cuvettes varied between 1 and 10 mW/cm^2 as measured with a thermopile (Kipp and Zonen, Holland). After 3–7 hr of illumination the cells were spun down, resuspended in 2.5 ml water with addition of 1 mg DNase and layered on top of a 25–45% (w/w) sucrose density gradient. The samples were then centrifuged for 17 hr at 130 000 *g* and the gradient was fractionated. Disc

* The high scattering of the samples did not introduce errors because control experiments where the turbidity of the samples was greatly reduced gave the same results. Scattering can be reduced by lysis of the cells through treatment with 0.5 mg/ml taurochenodesoxycholate (Calbiochem, California) and 1 mg/ml DNase (Roth OHG, Karlsruhe, Germany).

gel electrophoresis was carried out according to [8], thin-layer chromatography of retinaloxime as described in [7]. Retinaloximes were extracted in a solvent mixture ethanol–water–chloroform = 5:2:2.

Purple membrane suspensions in water, containing approximately 2×10^{-5} M bacteriorhodopsin and 0.2 M hydroxylamine were illuminated as described for the cell suspensions. Bleaching was complete after 1 3 hr, sucrose density gradient centrifugation (30–50% w/w) as described for cell lysates.

3. Results and discussion

Fig. 1 summarizes our present knowledge on the photochemical cycle in *H. halobium* cells. The purple complex in bacteriorhodopsin absorbs light (λ_{max} 560 nm) and is photochemically (i.e. temperature independently) converted to the 412-nm complex with concomitant proton release to the medium. The 412-nm complex reconverts in a temperature dependent process into the purple complex under proton uptake from inside the cell [4,5]. In the absence of hydroxylamine a photoequilibrium between the two states of the chromophore is established and the ratio of their concentrations in the steady state is given by:

$$\frac{[412]}{[560]} = \frac{k_1}{k_2} \times I \qquad (1)$$

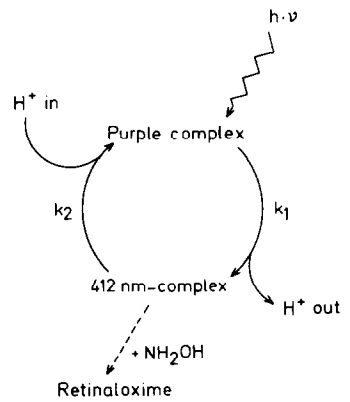

Fig. 1. Photochemical cycle in *H. halobium* mediating light energy conversion. H^+_{in} and H^+_{out} indicate the vectorial nature of this process.

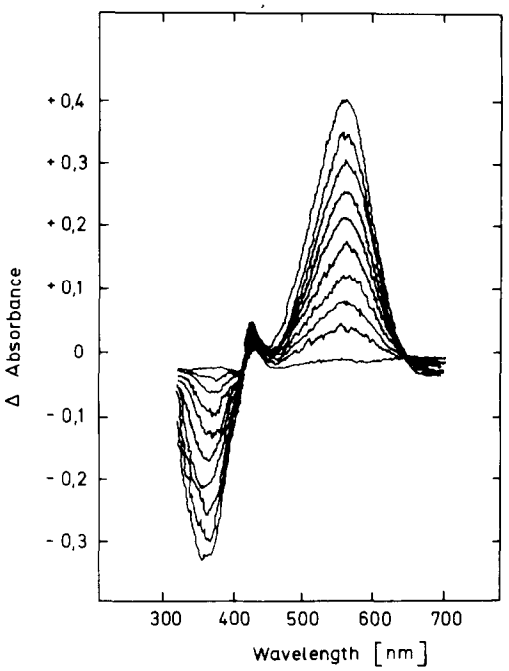

Fig. 2. Difference spectra of illuminated cell suspensions with and without hydroxylamine. Cells were concentrated 10-fold. (Concentration 1 is that of cells in the medium after 4 days growth.) NH_2OH concentration 0.2 M. Spectra were taken at: 0, 11, 23 35, 50, 66, 87, 104, 124 and 149 min. Positive values indicate decrease in absorption of the NH_2OH containing cuvette.

k_2 has values in the sec range if the cycle is observed using purple membrane suspensions in salt–ether mixtures [4]. The ratio k_1/k_2 is such that the 412-nm complex dominates the photoequilibrium even at low light intensities [4]. The reactions are much faster in water and in intact cells and the ratio k_1/k_2 is changed compared to the salt–ether system so that even high light intensity (I) produces only very small steady state concentrations of the 412-nm complex which are difficult to demonstrate. Coupling the photochemical reaction to the irreversible formation of retinaloxime indicated in fig. 1 allows chemical trapping of the 412-nm complex.

Suspensions of *H. halobium* R_1M_1 cells in basal salt appear purple colored due to bacteriorhodopsin as the predominant pigment absorbing in the visible region. Exposed to strong orange light in the presence of hydroxylamine they become pale yellow. The difference spectra in fig. 2 identify the components

involved in this photochemical reaction. The purple complex band (560 nm) of bacteriorhodopsin disappears and the retinaloxime absorption band appears (380 nm). The same result is obtained if both samples contain hydroxylamine but only one is illuminated. Retinaloxime as a reaction product is identified further by thin layer chromatography after extraction of the bleached cells with ethanol–chloroform.

The time course of the reaction is shown in fig. 3A indicating that in time all bacteriorhodopsin is converted into retinaloxime and bacterio-opsin. According to fig. 1 the rate of this reaction should be influenced by light intensity, by hydroxylamine and by bacteriorhodopsin concentration.

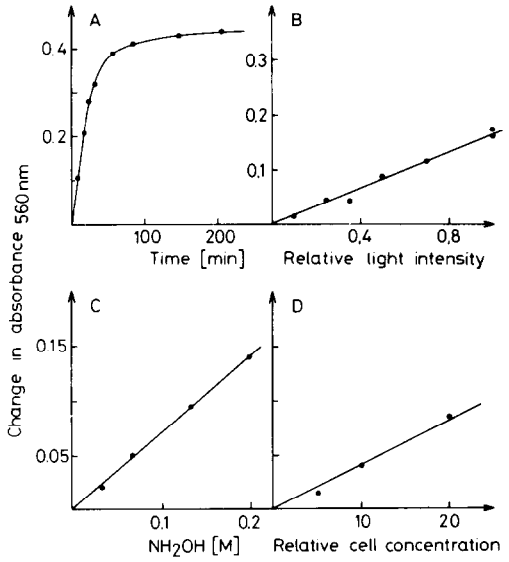

Fig. 3. Dependence of the NH_2OH-reaction on different parameters. A: Time dependence; cell concentration 10-fold NH_2OH concentration 0.2 M; light intensity 1; B: Light intensity dependence. Cell concentration 20-fold; the difference in absorbance after 20 min (linear part of the time curve) is plotted against relative light intensity. Intensity 1 is light as described under material and methods. It was weakened by calibrated neutral glass plates (Schott). NH_2OH concentration 0.2 M; C: Dependence on hydroxylamine concentration. Time curves of the reaction were taken at different NH_2OH concentrations and absorbance differences from its linear part (10 min) plotted. Cell concentration 10 fold; light intensity 1; D: Dependence on cell concentration. Time curves at 0.2 M NH_2OH concentration were measured and the absorbance differences from its linear part (10 min) plotted; light intensity 1.

Since the steady state concentration of the 412 nm-complex is given by:

$$[412] = \frac{[B]}{1 + k_2/k_1 \times I} \qquad (2)$$

the retinaloxime formation is

$$\frac{d\,[\text{Retinaloxime}]}{dt} = k_3 \frac{[B]}{1 + k_2/k_1 \times I} \times [NH_2OH] \qquad (3)$$

Fig. 3C and D demonstrate the expected linear dependence of retinaloxime formation on hydroxylamine and relative cell (i.e. bacteriorhodopsin) concentration under the conditions of initial rates (i.e. nearly unchanged reactant concentrations) and constant light intensity. The linear dependence on light intensity (fig. 3B) indicates that under our conditions of illumination the value of $k_1 \times I$ is negligible compared to k_2 (eq. 3).

These results obtained with intact cells agree well with the data obtained on the isolated purple membrane. This method also allows a quantitative determination of bacteriorhodopsin in cell cultures without prior isolation of the purple membrane (see also [6]). Furthermore, it can be shown that bleached cells, although viable after NH_2OH-treatment, do not show anymore the light induced pH-changes which accompany light energy conversion in *H. halobium*.

In order to answer the question whether bacterio-opsin shows the same tendency to form membrane patches as bacteriorhodopsin does, its location within the membrane surface was analysed. For this purpose aliquots of illuminated cell suspensions with and without hydroxylamine were exposed to pure water which causes lysis of the cells and fragmentation of the cell membrane [10]. The lysate was then applied to a sucrose density gradient and centrifuged.

Whereas the control (fig. 4A, band I) shows the typical purple membrane band (as identified by its characteristic 560 nm-absorption) in the lower part of the tube and no bacteriorhodopsin can be detected spectroscopically in other membrane fractions under the growth conditions used, the hydroxylamine-treated sample contains a white (no absorption at 560 nm) membrane fraction at the same position as

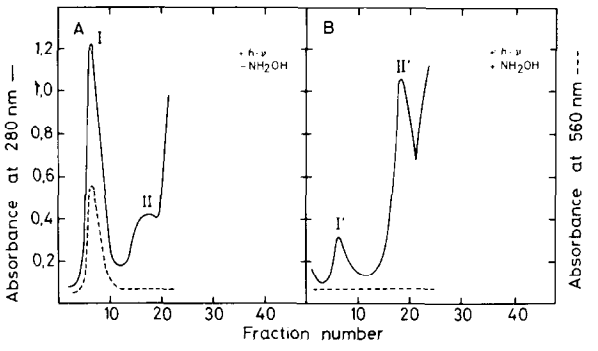

Fig. 4. Sucrose density gradient (25–45% w/w) of illuminated cells after lysis. A: Cell-suspensions without NH_2OH; B: With NH_2OH (0.2 M). Duration of illumination 7 hr for A and B.

the purple membrane but with a reduced amount of protein compared to the control (fig. 4B, band *I'*). It is obvious from fig. 4A and B that concomitantly with the decrease of protein content in band I (compared to *I'*) there is an increase in membrane fraction II' (compared to II). This band of the gradient is located just below the bulk membrane bands and the cytoplasmic components in the upper part of the gradient.

The protein composition of bands II and II' were analyzed by SDS-gel electrophoresis* and compared with the protein from bands I and I' (fig. 5). All four membrane bands contain exclusively bacteriorhodopsin (bacterio-opsin respectively)** and, qualitatively, the gel-stain reflects the inversion of protein content seen in the density gradient.

Therefore as a consequence of the conversion of bacteriorhodopsin into bacterio-opsin the latter moves from a membrane fraction of higher buoyant density (I/I') to another of lower density (II/II'). The same experiment carried out with isolated purple membrane in aqueous suspension does not result in a change of the buoyant density of the membrane but only in a

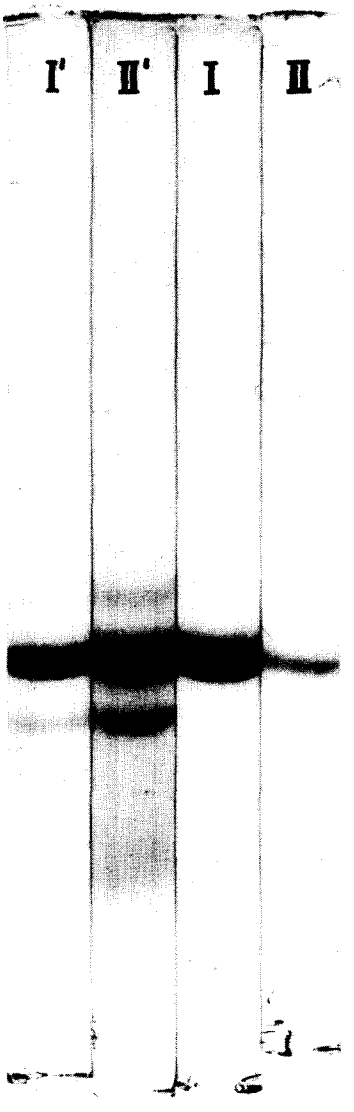

Fig. 5. Disc gel electrophoresis of membrane fractions from lysed cells in SDS. Cell lysates were fractionated as shown in fig. 4 and the bands I, II, I' and II' washed free of sucrose by centrifugation at 100 000 *g* for 1 hr. Samples were dissolved in equal volumes buffer as described in [8] and equal volumes applied to the gels (10%). The numbering of gels refers to figs. 4A and B. The two satellite bands on the gels I' and II' are not always observed and are unexplained so far.

disappearance of the 560 nm absorption (fig. 6).

We interpret these results in the following way: Bacteriorhodopsin forms a hexagonal crystalline lattice within the plane of the purple membrane [2], indicating strong interaction which preserves the mem-

* Only fractions 13 to 18 were combined in order to avoid contamination from material in the upper part of the gradient.

** When the purple membrane fractions are dissolved in SDS-buffer the chromophore is destroyed and retinal split off the protein, thus eliminating possible differences between bacteriorhodopsin and bacterio-opsin.

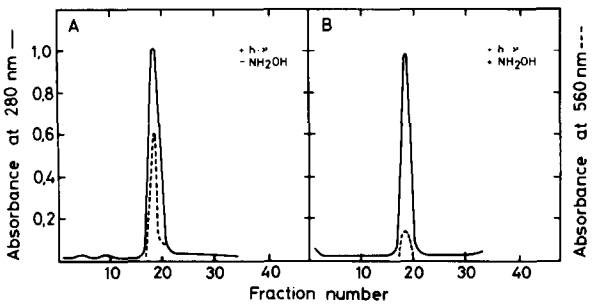

Fig. 6. Sucrose density gradient (30–50% w/w) of the purple membrane (A) and its apo-membrane (B). Duration of illumination 3 hr for A and B.

brane patches from disintegration during exposure to pure water. If bacterio-opsin would interact more weakly than bacteriorhodopsin it could diffuse latterally into other membrane regions or the former purple membrane patches could be disintegrated into smaller pieces. Both possibilities are excluded by the facts that in band II′ only bacterio-opsin is found and that this membrane fraction has a lower buoyant density. In addition, bacterio-opsin gives the chromophore-free membrane (fig. 6B) the same bouyant density as bacteriorhodopsin to the purple membrane. The most likely explanation for the existence of band II in the cell lysate is therefore a rearrangement of protein and lipid after conversion of bacteriorhodopsin into bacterio-opsin. In the case of the isolated purple membrane this process can not happen because the lack of extralipid in the aqueous phase. It should be mentioned that the movement of protein from band I to band II does not accompany the conversion of bacteriorhodopsin into bacterio-opsin but rather follows it. The data shown in fig. 4B are obtained after 7 hr of illumination. Complete bleaching is obtained already after 3 hr and if cells are lysed after that time most of bacterio-opsin is still found in band I.

As further evidence for the ability of bacterio-opsin to be a component of different membrane fractions we reconstituted bacteriorhodopsin in both, membrane fractions I′ and II′ by addition of retinal (see also [11]).

In analogy to the well-established nomenclature in enzymology we suggest to allocate the term apo-membrane and iso-apo-membrane for the chromophore free membrane and the membrane fraction of band II′ (fig. 4A) respectively. These analogous terms seem to be justified by the fact that the chromophore, like a co-enzyme, is necessary for the function in vivo. The reaction described in this paper provides a useful system of studying the interaction of a known membrane protein with lipids after its photochemically induced modification.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

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