

BIOSYNTHESIS OF PURPLE MEMBRANE: CONTROL OF RETINAL SYNTHESIS BY BACTERIO-OPSIN

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1. Introduction

The chemical composition of the purple membrane, a light-energy converting membrane system of halobacteria is surprisingly simple [1,2]. This differentiated domain of the plasma membrane contains only one protein species (bacterio-opsin), which is complexed with retinal in a 1:1 stoichiometry. The protein-retinal complex is called bacteriorhodopsin. For every bacteriorhodopsin molecule about ten lipid molecules are present in the purple membrane.

Biogenesis of purple membrane is inducible by limiting the oxygen supply [2], which turns on synthesis both of bacterio-opsin and of retinal [3]. In contrast, most of the lipid molecules necessary for purple membrane formation are synthesized long before the start of bacterio-opsin and retinal synthesis, i.e., the lipids are taken from the pool of the cell membrane [4].

Essentially all the retinal present in halobacteria is associated with bacterio-opsin, so one observes nearly stoichiometric relation between bacterio-opsin and retinal content. This fact suggests the existence of a highly efficient regulation mechanism coordinating the bacterio-opsin and retinal synthetic pathways. In this paper it is shown that bacterio-opsin itself is involved in the control of retinal production.

2. Materials and methods

2.1. *Halobacterium* strain and media

Halobacterium halobium R₁M₁ [5], which does not synthesize bacterioruberin, was used throughout and

grown in the peptone medium described in ref. [6]. Growth conditions inducing purple membrane formation were as follows: 500 ml Erlenmeyer flasks containing 230 ml medium were shaken with 100 reciprocating movements per min for 5 days at 40°C.

Low purple membrane production is observed under conditions of sufficient aeration: cells were grown in 500 ml Erlenmeyer flasks containing only 90 ml medium under otherwise identical conditions. Growth in the presence of 1 mM nicotine yielded cells with a high (230 ml medium) or low (90 ml medium) level of bacterio-opsin, respectively ('nicotine cells' [7]). Basal salt solution contained per liter 250 g NaCl, 20 g MgSO₄ · 7H₂O and 2 g KCl.

2.2. Retinal analogue

4-Ketoretinal was a gift from V. Christoffel. The compound was synthesized according to ref. [8].

2.3. Pigment extraction and quantitative determination

Retinal cells pelleted from 30–100 ml suspension were lysed in 1 ml water, containing DNAase. Under vigorous stirring, the lysate was dropped into 8 ml acetone. After 20 min in the dark, 4 ml *n*-hexane and 1 ml water were added. The upper phase, containing the pigments, was brought to dryness and the pigments were immediately taken up in 100 µl toluene. For the determination of retinal, an aliquot of this solution was applied as a streak on a thin-layer plate (silica gel 60, Merck). The plate was developed (about 10 min) with toluene/acetone 95:5. The yellow retinal band was cut out and immediately immersed in 600 µl ethanol. 200 µl of reagent 1 (4% thiourea in acetic acid) and 200 µl of reagent 2 (0.6%

thiobarbituric acid in ethanol) according to Futterman [9] were added. After stirring for 45 min in the dark, the absorption at 530 nm ($\epsilon = 58\,000\text{ M}^{-1}\text{ cm}^{-1}$) was measured with an Aminco DW 2 spectrophotometer.

3. Results and discussion

As shown recently, newly synthesized bacterio-opsin is initially incorporated in another differentiated domain of the cell membrane, that was called the brown membrane [3,7]. After complex formation with retinal, the bacteriorhodopsin still remains localized in this domain of the membrane. Assembly of bacteriorhodopsin within the membrane to yield the hexagonal-crystalline protein network of the purple membrane [10–12] commences only after a modification reaction. In brown membrane preparations isolated from halobacteria of the early stationary growth phase only about 80–90% of the bacterio-opsin present is complexed with retinal (i.e., 80–90% bacteriorhodopsin and 10–20% bacterio-opsin are present). This observation prompted us to speculate that the retinal synthesis might be activated by bacterio-opsin and therefore lags behind bacterio-opsin production. In order to examine this possibility, a retinal analogue was added to a growing culture of halobacteria in order to trap each newly synthesized bacterio-opsin molecule. The analogue used was 4-keto-retinal, which readily forms a complex with bacterio-opsin ($\lambda_{\text{max}} = 520\text{ nm}$ [13]). A culture grown under identical conditions but without addition of the analogue served as a reference. In fig.1 the absorption spectra of the purple membrane fractions isolated from these cultures are compared. The 'purple' membrane of the treated culture exhibits an absorption maximum at 520 nm with a weak shoulder at 570 nm, indicating that indeed most of the bacterio-opsin could be trapped by the externally added analogue. The main result of this experiment was the finding that the treated cells had a 3–4-fold lower retinal content than the reference cells, although the bacterio-opsin content was even slightly higher (10–20%) in the treated cells (as revealed by quantitative SDS-gel electrophoresis; not shown).

A control function of bacterio-opsin in the synthesis of retinal was further established by the

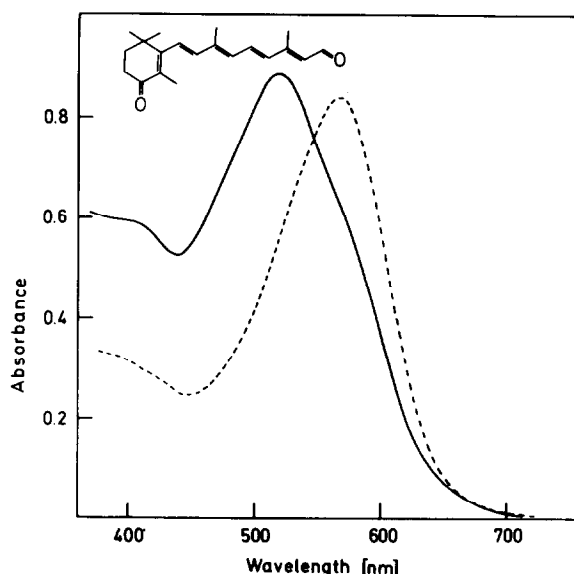


Fig.1. Absorption spectra of the purple membrane fractions isolated from cells grown in the presence (—) or absence (---) of the retinal analogue 4-keto-retinal. Cells were grown under limited oxygen supply (see Materials and methods) for 5 days. After 2 days of growth, every 10 h 4-keto-retinal (0.5 μmol in 50 μl *i*-propanol) was added to one culture.

following experiments. During the stationary growth phase and under conditions of limited oxygen supply, halobacteria continue to synthesize bacterio-opsin almost selectively [7]. Thus, the main effect of adding the protein synthesis inhibitor puromycin during this phase is a complete inhibition of bacterio-opsin synthesis, without affecting the viability of the cells. In the experiment of fig.2, puromycin was added to a culture in which the synthesis of retinal was well under way. As shown in fig.2 this inhibition of bacterio-opsin synthesis also causes the cessation of retinal synthesis.

Nicotine inhibits selectively the pathway of retinal synthesis by blocking the cyclization of lycopene to β -carotene [14]. Synthesis of bacterio-opsin, however, remains inducible in cells grown in the presence of nicotine (nicotine cells). Therefore nicotine cells grown under conditions of limited oxygen supply contain usually levels of bacterio-opsin 5-fold higher than do cells grown with sufficient aeration [7]. This fact was utilized to prepare cells with a high or low

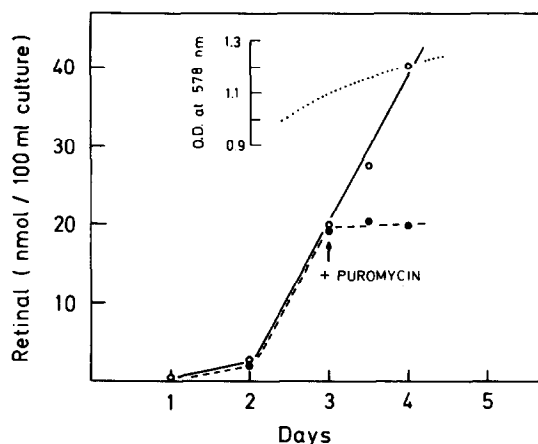


Fig. 2. Inhibition of retinal synthesis by puromycin. Cells were grown under conditions of limited oxygen supply (see Materials and methods). At the time indicated, one culture (----) was treated with puromycin (final concentration: 30 µg/ml), while another culture served as reference (—). Retinal content of the cells was determined as described in Materials and methods. The insert shows the absorbance at 578 nm of the reference culture.

bacterio-opsin level, respectively. At zero time, the retinal synthesis inhibitor nicotine was removed and both cell suspensions were further incubated in the presence of puromycin (to inhibit a further production of bacterio-opsin). As shown in fig. 3, retinal synthesis starts immediately in both cell suspensions. However, the extent of retinal production depends on the bacterio-opsin content of the cells. Synthesis levels off as soon as all the bacterio-opsin is converted to bacteriorhodopsin, as can be followed by the 570 nm chromophore formation (data not shown). This fact is clearly demonstrated by the experiment of fig. 3, curve 3: the nicotine cells containing a high level of bacterio-opsin were treated at zero time with 4-keto-retinal to convert all their bacterio-opsin to the bacteriorhodopsin analogue. After this conversion, these cells do not synthesize retinal any longer (curve 3).

The predominant carotenoid in nicotine cells is lycopene. In order to find out which step in the lycopene → β -carotene → retinal pathway is controlled by bacterio-opsin, nicotine cells were freed from nicotine and incubated under a nitrogen atmosphere [15]. Under these conditions nicotine

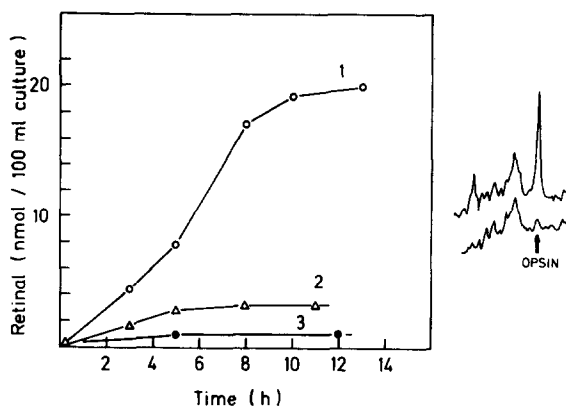


Fig. 3. Kinetics of retinal synthesis in cells containing different levels of bacterio-opsin. Nicotine cells were washed and resuspended ($A_{578} = 1.6$) in basal salt solution containing 0.5% L-alanine and puromycin (30 µg/ml). Cell suspensions were shaken at 40°C. Curve 1: nicotine cells with high bacterio-opsin content (see Materials and methods). Curve 2: nicotine cells with low bacterio-opsin content. Curve 3: conditions as for curve 1, except for the addition of 4-keto-retinal (15 nmol/ml) at zero time. The scans of SDS-polyacrylamide gel patterns demonstrate the different bacterio-opsin levels of the cells used.

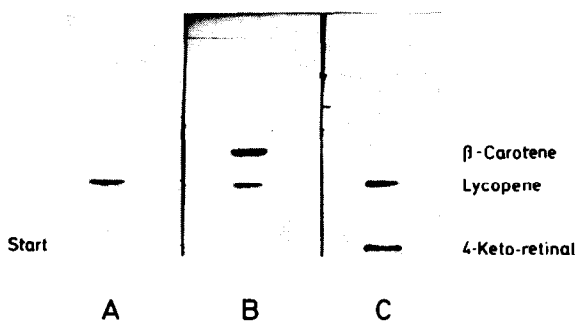


Fig. 4. Thin layer chromatogram of pigments synthesized in nicotine cells containing different levels of bacterio-opsin. Nicotine cells were washed and resuspended ($A_{578} = 1.5$) in basal salt solution containing 0.5% L-alanine. Incubation was at 40°C for 20 h under a nitrogen atmosphere. A: pigment analysis before incubation. B: pigment pattern after incubation. C: as for B, except for the addition of 20 µmol/ml 4-keto-retinal at zero time. Extraction of pigments as described in Materials and methods. Plates were developed with *n*-hexane/ether (98:2) and stained with the Carr-Price reagent.

cells convert most of their lycopene to β -carotene (fig.4B). Synthesis of retinal is not possible, owing to the oxygen requirement of the β -carotene \rightarrow retinal reaction. In the experiment of fig.4 C the bacterio-opsin of the nicotine cells was converted to a bacteriorhodopsin analogue by the addition of 4-keto-retinal at zero time. As shown in fig.4 C, the conversion of lycopene to β -carotene is now blocked, indicating that bacterio-opsin is involved in the control of this step.

The experiments described demonstrate clearly a control function of bacterio-opsin in retinal synthesis and this result explains the coordinated synthesis of bacterio-opsin and retinal during purple membrane biosynthesis. These in vivo experiments, however, cannot distinguish between two alternative interpretations. Either bacterio-opsin (but not bacteriorhodopsin) activates the (membrane bound?) enzyme system catalyzing the cyclization of lycopene or retinal itself is a strong feedback inhibitor of this enzyme system. In the latter case, bacterio-opsin would control indirectly the extent of retinal synthesis: as soon as all the bacterio-opsin present is converted to bacteriorhodopsin a slight excess of retinal would also turn off the cyclization of lycopene.

Acknowledgement

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