Effects of Light and Low Oxygen Tension on Pigment Biosynthesis in *Halobacterium salinarum*, Revealed by a Novel Method to Quantify Both Retinal and Carotenoids

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A novel method for analyzing halobacterial pigments was developed, in which retinal was liberated from halobacterial rhodopsins as retinal oxime by hydroxylamine, ethyl β-apo-8'-carotenoate was introduced as an internal standard, and the pigments including bacterioruberin and β-carotene were analyzed by HPLC at the same time. With this method, we revealed that light enhances the biosynthesis of bacterioruberin and the conversion of β-carotene to retinal, but does not affect β-carotene biosynthesis in *Halobacterium salinarum* strain Oyon Moussa-16. Low oxygen tension given in the light brought a slight increase in retinal accumulation, although its biosynthesis from β-carotene is an oxygenation reaction. This paradox could be explained by the increase in β-carotene biosynthesis.

Key words: Bacterioruberin — Hydroxylamine — Low oxygen tension — Retinal oxime — Total β-carotene biosynthesis.

Abbreviations: loc, low organic components; TBS, total β-carotene synthesis.

Introduction

Halophilic archaea of the family Halobacteriaceae grow chemooorganotrophically in the dark. While in the light, they can utilize light energy, though still depending on organic nutrients as carbon source. The molecule responsible for the light utilization is bacteriorhodopsin that functions as a proton pump to generate ATP through cis-trans isomerization of the chromophore, retinal. Besides bacteriorhodopsin, halophilic archaea have three other retinal-binding proteins, namely, halorhodopsin, sensory rhodopsin and phorborhodopsin (Mukohata et al. 1999); bacteriorhodopsin is most abundant in the four. *Halobacterium salinarum* has two major carotenoids, bacterioruberin and β-carotene, both of which are thought to be synthesized from lycopene (Sumper et al. 1976). Bacterioruberin is a C30' xanthophyll functioning for photo-protection (Dundas and Larsen 1963, Shahmohammadi et al. 1998) while β-carotene is the precursor to retinal. Thus, the pigments of photoprotection and photo-utilization are synthesized from the same precursor. We are interested in biosynthetic regulation for these pigments. However, retinal is bound to the apoproteins forming Schiff bases, and thus, organic solvents cannot fully extract it, which has been an obstacle in the study of pigment biosynthesis in halobacteria.

Oesterhelt et al. (1974) showed that hydroxylamine liberates retinal from bacteriorhodopsin in the form of retinal oxime. However, this technique has not been utilized in analysis of total halobacterial pigments in a sophisticated manner. In the present study, we developed a novel HPLC method using hydroxylamine to quantify retinal as well as the carotenoids, and studied the effects of light and low oxygen tension on the pigment biosynthesis in H. salinarum.

Results

Use of dilute organic nutrients in halobacterial culture for pigment analysis

Prior to pigment analysis, we examined the effects of organic nutrient concentration on the growth of *H. salinarum*, since we suspected that excess organic nutrient might be used as an alternative energy source, thereby masking the effects of light on some cellular metabolisms. When the cells were cultured in the dark, they grew with shorter lag times and higher stationary level in the medium 166 HMg7-l than in the medium 166 HMg7 (Fig. 1). Light elevated the stationary level in the former medium, but not in the latter. This finding suggests that too much organic nutrient rendered the cells unhealthy and masked the potential effects of light on the growth. From this result, we adopted the conditions of one-tenth organic nutrients to study the effects of light on pigment biosynthesis in *H. salinarum*. The absorption spectra of the pigments extracted from the light- and dark-grown cells indicated that pigment composition largely changes depending on the light conditions (Fig. 2).
Novel method for analyzing halobacterial pigments

Conditions for pigment extraction

Although acetone is often used to extract halobacterial pigments (Kelly et al. 1970), this solvent was found to react with hydroxylamine, which was used to extract retinal; so, in this study we used chloroform/methanol instead. For efficient extraction of retinal as retinal oxime, we tried various concentrations of hydroxylamine. In the range of the concentrations from 3 to 45 mM, the extraction was nearly 98% and repeated extraction was practically unnecessary, based on colorlessness of the precipitate and quantification of retinal oxime from the precipitate by second extraction. The extraction of retinal oxime in the presence of hydroxylamine was more than three-fold that of retinal in the absence of hydroxylamine on a molecular basis.

Identification and quantification of pigments

Fig. 3 shows an example of an elution profile on HPLC of the pigments from the dark-grown cells. We identified the first peak as retinal oxime, based on the retention time and broad absorption peak at 360 nm, both of which were observed with hydroxylamine-treated standard. The syn and the anti forms of retinal oxime, which can be separated by normal phase HPLC (Oesterhelt et al. 1973), were not separated in our reverse phase HPLC system. We identified the second peak as bacterioruberin, based on its absorption peak at 490 nm and the relative molecular mass of 740 after isolation. The third peak was ethyl \( \beta \)-apo-8’-carotenolate, a known amount of which was added to the sample as internal standard upon extraction. The fourth peak was \( \beta \)-carotene, based on the retention time, absorption spectrum, the relative molecular mass of 536, and comparison with hydroxylamine-treated standard. The peak area of the halobacterial pigments was normalized with the internal standard taking account of the absorption coefficient at the absorption maximum of each pigment, which enabled direct comparison of the pigment contents among differently cultured cells. The presence of hydroxylamine upon extraction did not affect the retention times and absorption spectra of the carotenoids. Thus, this procedure enabled the simultaneous quantification of retinal, bacterioruberin and \( \beta \)-carotene. Some of the bacterioruberin and \( \beta \)-carotene changed to cis forms during preparation.
of samples, which eluted just after or before the main peaks of the all-trans forms, respectively (Fig. 3). The occurrence of the cis forms was unavoidable in the present procedure. We quantified these pigments assuming that the absorption coefficients of the cis forms are the same as those of the all-trans forms.

**Effects of light and low oxygen tension on pigment composition**

Table 1 shows the contents of the pigments in differently cultured cells of *H. salinarum*. The three pigments were found in the dark-grown cells; light increased retinal and bacterioruberin, but decreased β-carotene accumulation, which in total resulted in more than 80% increase in total carotenoids. No lycopene was detected in the cells grown under these conditions, although it was accumulated when the cells were cultured in the presence of an inhibitor of carotenoid biosynthesis, nicotine (Sumper et al. 1976). Since β-carotene is postulated to be cleaved at the center of the molecule to produce two retinal molecules (von Lintig and Vogt 2000, Wyss et al. 2000), we introduced the index “β-carotene + 0.5× retinal” to evaluate total β-carotene synthesis (TBS). TBS values were similar in the dark- and light-grown cells (approximately 5 nmol per 10^10 cells), suggesting that β-carotene biosynthesis is not influenced by light under the conditions used. Therefore, the increase in retinal by light must indicate the enhancement of the conversion of β-carotene to retinal. The similar values of TBS also indicate that the bacterioruberin biosynthesis is essential in the increase in the total carotenoids by light.

It is well known that limited oxygen supply causes accumulation of bacteriohopdsin in a bacterioruberin-less mutant of *H. halobium* (Sumper et al. 1976). This phenomenon suggests that retinal chromophore also accumulates under low oxygen tension. However, this is a paradox since molecular oxygen is a substrate of the reaction that forms retinal from β-carotene, and thus, this reaction should be suppressed by low oxygen tension. We questioned whether retinal really increases under low oxygen tension, and examined the pigment composition of the cells that were subjected to N_2 bubbling. This treatment is also expected to decrease bacterioruberin since a series of hydroxylation reactions (introduction of oxygen atoms) are involved in the biosynthetic process from lycopene to bacterioruberin (Kushwaha and Kates 1979). In the light-grown cells, the N_2 bubbling largely decreased bacterioruberin as expected, but slightly increased retinal. This finding suggests that there must be an unknown mechanism that indirectly promotes the retinal biosynthesis under low oxygen tension (see Discussion). In the dark-grown cells, the total carotenoid content was not much changed by N_2 bubbling. This fact suggests, referring to the postulated scheme of biosynthesis of halobacterial pigments (Fig. 4), that the carotenoid biosynthesis to lycopene is not influenced by oxygen decrease in the dark. Thus, the site of the primary event by oxygen decrease is likely to be located after lycopene, probably the suppression of bacterioruberin biosynthesis. The resulting surplus of the precursor lycopene would increase TBS.

**Discussion**

In the present study, we first optimized the conditions of organic nutrients to investigate the effects of light on the pigmentation of *H. salinarum*. The archaeon grew better in liquid culture at one-tenth the original concentration of the organic nutrients in the medium 166 HMG7, indicating that the higher
concentrations of organic nutrients are not necessarily better for the archaeal growth, as has been shown for other microorganisms (Seki 1992). It should be noted that the one-tenth concentration corresponds to the dissolved organic carbon of 800 mg liter$^{-1}$, which is still higher than those at natural habitats of extremely halophilic archaea, which are less than 100 mg liter$^{-1}$ (Saida et al. unpublished).

We introduced the TBS index to evaluate β-carotene biosynthesis in *H. salinarum*, assuming that β-carotene is cleaved at the center of the molecule to generate two molecules of retinal. This one-to-two stoichiometry is based on the recent finding of the reaction of β-carotene 15,15′-dioxygenase of higher organisms (von Lintig and Vogt 2000, Wyss et al. 2000). Although this enzyme has not been found in halophilic archaea, the same stoichiometry is widely believed since no intermediates in other potential processes from β-carotene to retinal have been reported to date. Furthermore, the cleavage of β-carotene at the center of the molecule is the only biosynthetic pathway for retinal ever described in any organism.

Fig. 4 summarizes the results of the present study, together with a previously revealed mechanism of regulation of halobacterial pigment biosynthesis (Deshpande and Sonar 1999, Oesterhelt and Stoeckenius 1973, Shand and Betlach 1991). This study revealed that light enhances the conversion from β-carotene to retinal, but the mechanism of the enhancement remains unknown. A plausible hypothesis is that the photoreceptor is β-carotene and its possible structural change promotes the interaction with the cleaving enzyme. An alternative is that the photoreceptor would be the enzyme itself. It would be interesting to learn whether the cleavage reaction of β-carotene 15,15′-dioxygenase of higher organisms is also enhanced by light.

Light also enhanced the biosynthesis of bacterioruberin that is postulated to function in photoprotection (Dundas and Larsen 1963, Shahmohammadi et al. 1998). Recent studies on bacterial and plant photosynthesis indicate that (bacterio) chlorophyll–protein complexes are necessarily accompanied by carotenoids for photoprotection. Thus, it appears reasonable that the biosynthesis of a pigment for photoprotection (bacterioruberin) is enhanced in parallel with that for photo-utilization (retinal). Since TBS was not changed by light under the normal oxygen conditions, the increase in the total carotenoid biosynthesis reflects that in bacterioruberin (Table 1), suggesting that the net carotenoid biosynthesis is for photoprotection. This mechanism may imply that security precedes efficiency in the light energy utilization in the halobacterial biosystem.

This study showed that retinal was increased by low oxygen tension, although the biosynthesis of retinal must require molecular oxygen as a substrate. A key to solve this paradox may be the regulation of biosynthesis of the apoprotein of bacteriorhodopsin, bacterioopsin; Shand and Betlach (1991) found that expression of a bacterioopsin gene was stimulated by low oxygen tension, and Deshpande and Sonar (1999) revealed that bacterioopsin protein introduced into halobacterial cells in proteoliposomes induced the conversion of lycopene to β-carotene. Thus, low oxygen tension should increase the β-carotene supply for retinal biosynthesis. In fact, in the present study, TBS increased under low oxygen tension. We interpret that low oxygen tension influences the retinal biosynthesis in two ways, direct suppression through the substrate (oxygen) decrease and indirect promotion by the increase in β-carotene supply through the promotion of bacterioopsin biosynthesis (Fig. 4). Another possible mechanism of the increase in retinal under low oxygen tension is that some fraction of retinal would be continuously destabilized by molecular oxygen under the atmospheric concentration of molecular oxygen. A decrease in oxygen tension would retard the destabilization, which should result in the accumulation of retinal, together with stabilization of retinal through binding to bacterioopsin, whose biosynthesis is enhanced by low oxygen tension (Shand and Betlach 1991). Further study is needed to clarify the mechanism by which retinal is accumulated under low oxygen tension. Many things remain to be done for comprehensive understanding of the mechanism of regulation of halobacterial pigment biosynthesis, but we expect that the present study will provide a potent technique as a first step toward this goal.

**Materials and Methods**

**Strain and culture**

*Halobacterium salinarum* strain Oyon Moussa-16 (Saida et al. 2001), deposited in the Japan Collection of Microorganisms as JCM 10927, was used in this study. The strain was maintained at room temperature on agar slants containing 25% NaCl, 1.5% Bacto-casamino acids, 0.3% trisodium citrate, 0.25% glutamic acid, 2.0% MgSO$_4$·7H$_2$O, and 0.2% KCl, pH 7.0 (termed medium 166 HMg7); the concentration of MgSO$_4$·7H$_2$O and pH in this medium were modified from JCM medium 166 (http://www.jcm.riken.go.jp/JCM/JCM_GRMD.html). For liquid culture, the concentrations of the three organic components were reduced to one-tenth: 166 HMg7-long organic components (-loc). The cells were cultured in 250 ml of medium 166 HMg7-loc in a 500-ml Erlenmeyer flask with gentle stirring at 37°C in the light (60 μmol photons m$^{-2}$ s$^{-1}$ from fluorescent lamps) or in the dark. These conditions provide incomplete aeration to induce biosynthesis of purple membranes (Oesterhelt and Stoeckenius 1973). The flask was plugged with cotton, through which a silicon tube (i.d. 4 mm) ran into the medium. A filter unit of cellulose acetate with a pore size of 0.2 μm (DISMIC-25cs; ADVANTEC, Tokyo) was attached to the outside end of the tube. For some cultures, N$_2$ gas was supplied through the filter unit for 1 min to further decrease oxygen tension 1 d before harvest. After the N$_2$ bubbling, the tube was removed and the flask was sealed with Parafilm M (American National Can, Chicago). The oxygen concentration before and after the bubbling was about 70 and 30 μM, respectively, when measured with a Clark-type oxygen electrode (DW1; Hamsatech, King’s Lynn) at 25°C. A similar concentration to the latter was observed when measured upon harvest. At harvest, an aliquot of culture was taken for measurement of cell density with a phase contrast microscope (Eclipse E600; Nikon) using a bacterial counting chamber. The cells were harvested at the early stationary phase by centrifugation at 7,000g for 20 min. They were washed twice with 4 M NaCl, and stored at −80°C until use in 1.5-ml sample tubes containing known cell numbers from 4 to 8×10$^7$ cells.
Pigment analysis

Analysis of pigments was performed under dim light, as follows. The frozen cell pellet was thawed and 20 µl of 1 M hydroxylamine-HCl in 1 M Tris-HCl (pH 8.0), 1.5 ml of chloroform/methanol (2:1, v/v), and 10 µl of 0.5 mM ethyl β-apo-8'-carotencate (trans; Fluka) in ethanol were added. The suspension was sonicated with a sonic oscillator (VP-5S; TAIITEC, Koshigaya, Japan) for several s and centrifuged. The supernatant was collected and evaporated. The pigments were dissolved in a small volume of chloroform/methanol (3:1, v/v) and an aliquot was subjected to pigment analysis by HPLC with a µBondapak C18 column (8x100 mm, RCM type; Waters) as described previously (Takaichi and Ishidzu 1992). The elution was performed with methanol/water (95:5, v/v) for the first 10 min and thereafter with methanol at a flow rate of 2 ml min\(^{-1}\). Absorption spectra were recorded with a photodiode-array detector (250–580 nm; 1.3 nm intervals; MCPD-3600; Otsuka Electronics, Osaka) attached to the HPLC apparatus (Takaichi and Shimada 1992).

The peaks of β-carotene and retinal oxime were identified by eluting standard β-carotene (Wako, Osaka) and all-trans retinal (Merck) after treatment with hydroxylamine as above. The absorption maxima of retinal oxime, bacterioruberin, the internal standard of ethyl β-apo-8'-carotenolate, and β-carotene were at 360, 490, 445, and 445 nm, respectively, in the HPLC eluents used. For quantification of these pigments, we determined the absorption coefficient of retinal oxime to be 60 mM\(^{-1}\) cm\(^{-1}\) at 360 nm, based on that of all-trans retinal. The absorption coefficients of bacterioryberin at 490 nm and of β-carotene at 445 nm were assumed to be 167 mM\(^{-1}\) cm\(^{-1}\) (Kelly et al. 1970) and 140 mM\(^{-1}\) cm\(^{-1}\) (Davies and Köst 1988), respectively. That of ethyl β-apo-8'-carotenolate at 445 nm was assumed to be 100 mM\(^{-1}\) cm\(^{-1}\), being assumed to be the same as that of β-apo-8'-carotenolic acid (Isler et al. 1959). For identification of the elution peaks, relative molecular mass was determined by field-desorption mass spectrometry using a double-focusing gas chromatograph/mass spectrometer equipped with a field desorption apparatus (M-2500; Hitachi) as described previously (Takaichi 1993).

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Reference


