

Biosynthesis of the Two Halobacterial Light Sensors P₄₈₀ and Sensory Rhodopsin and Variation in Gain of Their Signal Transduction Chains

JUN OTOMO,¹ WOLFGANG MARWAN,¹ DIETER OESTERHELT,^{1*} HERBERT DESEL,² AND RAINER UHL²
Max-Planck-Institut für Biochemie, 8033 Martinsried,¹ and Max-Planck-Institut für Biophysikalische Chemie, 3400 Göttingen,² Federal Republic of Germany

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The two retinal-containing photoreceptors of halobacteria, P₄₈₀ and sensory rhodopsin, are formed constitutively and inducibly, respectively. Both photoreceptors are synthesized as apoproteins in cells with nicotine-inhibited retinal synthesis and are reconstituted as chromoproteins by the addition of all-trans retinal to cell membrane preparations. The decrease in photoreceptor-mediated photophobic response at the stationary growth phase of cells is not due to photoreceptor degradation but due to a deficiency of the signal transduction chain in the cell.

Halobacteria thrive phototrophically in brines with saturating concentrations of sodium chloride and other salts. The two light-driven ion pumps bacteriorhodopsin and halorhodopsin absorb light in the visible range of 500 to 600 nm by their retinal chromophores and translocate protons and chloride ions to the outside or the inside of the cell, respectively (for a review, see reference 5).

Halobacteria have also developed two light-sensing systems, PS₅₆₅ and PS₃₇₀, for finding photosynthetically efficient green to orange light and avoiding inefficient blue or dangerous near-UV light (4). Without a stimulus, the cell carries out a random walk around a center constant in space (7). If a stimulus of orange light reaches the cell, it reacts with a prolonged run and does the opposite upon stimulation with light below 500 nm in the presence of orange background light (12). This results in a movement towards the orange or away from the blue light source.

The molecular basis of the two light-sensing systems was found only recently when a third retinal protein was characterized spectroscopically in halobacterial cells (1, 18). This protein, called sensory rhodopsin (SR, synonymous to SR-I), has photochromic properties and was postulated to mediate both the orange-light response by its dark-state SR₅₈₇ and the blue-light response by its photochemically produced intermediate, SR₃₇₃ (11). This concept did not explain, however, photophobic responses of halobacterial cells in the absence of orange background light in which no SR₃₇₃ is formed, and within a short time the existence of a fourth pigment, P₄₈₀ (synonymous to SR-II, phoborhodopsin), was demonstrated by several groups (7, 16, 20). Here, we report on the time course of biosynthesis of the two sensors, P₄₈₀ and SR, during growth and their changing activity in cells due to variation in gain of the signal transduction chain controlling the flagellar motor switch of the cell.

MATERIALS AND METHODS

Halobacterium halobium Flx37 (BR⁻ HR-deficient [8% RUB⁻] (10) and Flx15 (BR⁻ HR⁻ RUB⁺) (15) were grown in a 10-liter fermenter under standard conditions (9) or in 100-ml Erlenmeyer flasks containing 35 ml of medium on a gyratory shaker (100 rpm). Unless indicated otherwise, cells

were grown in 10-liter fermenter batches. Nicotine (2 mM) (14) was added to the culture medium if retinal synthesis was to be prevented. Samples were taken from the culture at various times, and the photoresponses were measured as described elsewhere (7). In short, 5 μ l of a cell suspension was put onto a precleaned slide and covered with a slip (20 by 20 mm). Measurements were taken after 5 min in the dark at 21°C (\pm 1°C). Actinic light was generated by a mercury lamp (Osram HBO, 100 W), and the 366-nm (SR) or the 436-nm (P₄₈₀) line was selected by interference filters. For the assay of SR, but not of P₄₈₀, constant orange background light (irradiance = 150 W/m²; cutoff filter OG 570) was present. The actinic light was passed through an epillumination attachment (Ploemopak Leitz/Opto-Metric Div. of E. Leitz Inc.) and was applied as pulses of 250 or 500 ms duration. Except for this change, the microscopical setup for observation of cellular stop responses in infrared light ($\lambda \geq 780$ nm) was as described previously (7). For each datum point, 30 individual responses were averaged and the stimulus-response curve was recorded as dependence of response time (t_R) on photon exposure (F). The slope of the straight line obtained by plotting t_R versus $1/F$ is called relative quantum efficiency and was used as a measure of sensory activity (see Fig. 1; for derivation of this plot see reference 7).

For measurements of stochastic responses of Flx37 cells to flashes of light, exactly the same experimental setup was used, but actinic light (366 nm) was decreased to a range in which cells would either neglect the stimulus or react with the maximum response time (t_{max}) (7). The irradiance at constant flash time was varied, and the percentage of responding cells was evaluated from 60 to 90 individual events. Each cell was stimulated only once. The obtained data were fitted to theoretical Poisson stimulus-response curves by using a least-square fitting routine.

Pigment concentrations were determined by harvesting the cells at distinct times and by preparing a total membrane fraction as described previously (13), followed by fast difference spectroscopy (19). From cells grown in the presence of nicotine, the same membrane fraction was prepared and the pigments were reconstituted by the addition of all-trans retinal in isopropanol (45 μ M) to a final concentration of 450 nM. The preparation of a Tween-washed membrane was as

* Corresponding author.

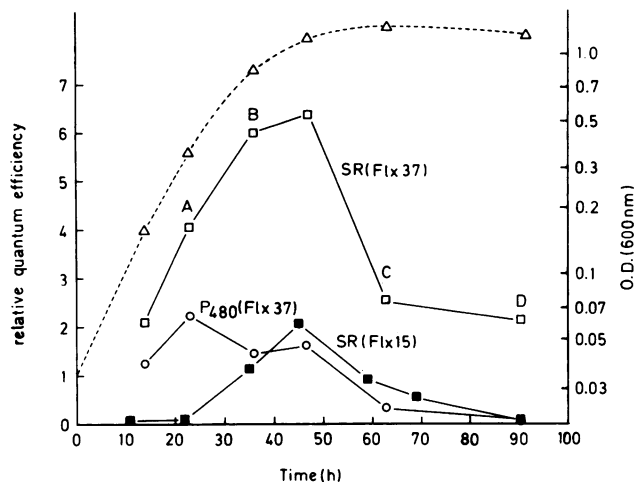


FIG. 1. Time course for the activities of P_{480} and SR during growth of *H. halobium* Flx37 and Flx15. Receptor activities were determined as described in Materials and Methods, and growth followed by turbidity changes was measured at 600 nm (Δ , log scale). Times at which receptor concentrations were determined (A to D) by difference spectroscopy on isolated cell membranes are indicated (see Fig. 2).

described previously (13) but with a protein:detergent ratio of 1:3. Halorhodopsin activity was determined as described elsewhere (8).

RESULTS AND DISCUSSION

Figure 1 shows the cellular activity of P_{480} and SR in *H. halobium* Flx37 and Flx15 during growth. The cells were observed with infrared light, and the activity of P_{480} was measured by the time a cell requires for a stop response after a flash of blue light (wavelength, 436 nm) in the absence of orange background light. Upon stimulation with flashes of increasing intensity, this response time (t_R) (i.e., the mean value of the response time distribution) decreases according to the equation (see reference 7) $t_R = (b/F) + t_{min}$, where $F = I \cdot \tau$ is the photon exposure, I is irradiance, τ is the flash time, and t_{min} is the response time at infinite irradiance. The constant b is related to properties of the photoreceptor, such as quantum yield, molar extinction, and concentration, but also to the gain of the signal transduction chain connecting the photoreceptor with the final target of the stimulus, i.e., the flagellar motor. The minimal response time (t_{min}) reflects the thermal rate-limiting step of the signal transduction chain which can be due to a low concentration of either the photoreceptor itself or a component of the chain. The plot of t_R versus $1/F$ yields a straight line, and the inverse value of its slope is called relative quantum efficiency and was plotted in Fig. 1 as a function of cell growth.

The photophobic response mediated by the activity of SR is also measured as the stimulus intensity-dependent response time t_R , but the stimulating light had a wavelength of 366 nm and the cells were continuously illuminated with orange background light to produce a sufficient SR_{373} photoreceptor pool. No difference was found in the behavior of different individual cells. In Flx37 cells, P_{480} -mediated activity was essentially constant until the beginning of the stationary phase (50 h). During the same time, SR-mediated activity increased by a factor of at least 3. This indicates that P_{480} is made by the cells constitutively but that SR, like the two ion pumps bacteriorhodopsin and halorhodopsin, is

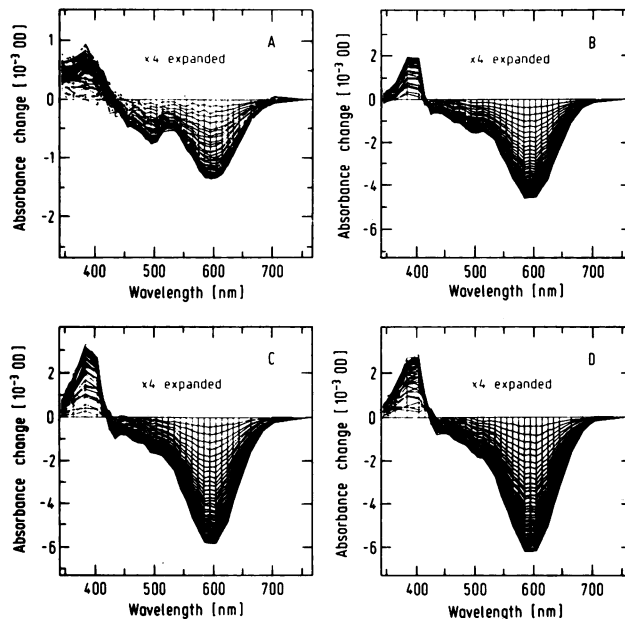


FIG. 2. Difference spectra of membrane fractions from Flx37 cells after 23 (A), 36 (B), 63 (C), and 91 (D) h of growth. The spectra were recorded at room temperature in 10-ms time intervals between 0 and 500 ms after the white light used simultaneously for actinic action and for monitoring absorbance changes was turned on. Cell membrane fractions were suspended in 4 M NaCl, and sample concentrations were 1.5 optical density units at 400 nm.

induced presumably by the dropping oxygen tension in the growing culture. This induction is more clearly demonstrated for Flx15 cells (Fig. 1), which, up to 24 h, showed no measurable SR activity at all but also reached a maximum at 50 h.

P_{480} and SR activities decreased in the stationary phase (Fig. 1, after 50 h). This could be due either to a decrease in photoreceptor concentration or to a decrease in gain of the signal chain. We therefore isolated membranes from cells of various growth stages (Fig. 1, datum points A to D) and analyzed the photoreceptor concentration by fast scanning spectroscopy (19). Figure 2A to D shows a time series of the spectra after the white light of the measuring beam was turned on (Fig. 2A to D correspond to the equally assigned datum points of Fig. 1). Spectra were recorded every 10 ms, and the continuous rise of photostationary-state levels of intermediates absorbing at 360 to 380 nm (positive absorbance change) and the depletion of the respective ground states are demonstrated (Fig. 2). A final stationary state was reached in 400 (P_{480}) and 500 ms (SR). At this time all molecules are presumably converted into the intermediate states because additional experiments, in which the intensity of the measuring beam was lowered, did not decrease the amount of intermediate or ground-state bleaching. Therefore, the amplitudes at 480 and 590 nm in the difference spectra could be used to calculate the respective number of P_{480} and SR molecules per cell. The extinction coefficient ($\epsilon = 40,000$) used for calculation is that of retinal and is the lowest estimate for retinal proteins (2). In the 23-h culture, $430 \pm 50 P_{480}$ molecules per cell were found, and $4,000 \pm 500$ SR molecules per cell were found in the 91-h culture. The white light used for establishing the photostationary state could conceivably lower the concentration of the intermediates by photochemistry of its blue-absorbing intermediate. A

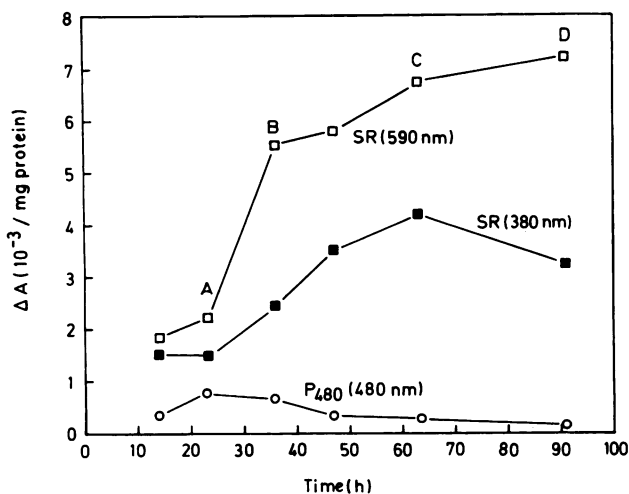


FIG. 3. Absorbance changes of membranes normalized to 1 mg of total membrane protein from cells of different ages grown in a 10-liter fermenter. The apparent decrease of P₄₈₀ might be artificial, due to difficult evaluation of the complex spectra in Fig. 2. Times at which absorbances were determined (A to D) are indicated.

control experiment with orange light instead of white light was carried out, and its result demonstrated no change in the stationary amplitudes.

No measurable P₄₈₀ or SR concentration was found in the cytoplasmic supernatants of the membrane pellets. The membranes from cells after 24 h of growth contained comparable amounts of P₄₈₀ and SR, but in time SR increased by a factor of almost 4, whereas P₄₈₀ stayed constant and was seen in the difference spectra of samples from older cells only as a shoulder (Fig. 2C and D). The constant level of P₄₈₀ in growing cells was also reported elsewhere (17). To quantify the effect seen in the experiment, the changes of photoreceptor concentrations were measured at more time intervals (Fig. 3). The most remarkable result of these experiments can be seen in the rise of SR concentration during growth to a constant level, clearly indicating that the drop in sensory activity during the stationary phase is due to the decay of signal chain components of the cell. The motility itself, i.e., the flagellar motor and the flagella, was not affected.

For separation and isolation of the receptors it is essential to know not only their localization but also the properties of these membrane-bound proteins with respect to detergent-mediated solubility. Figure 4 shows the removal of P₄₈₀

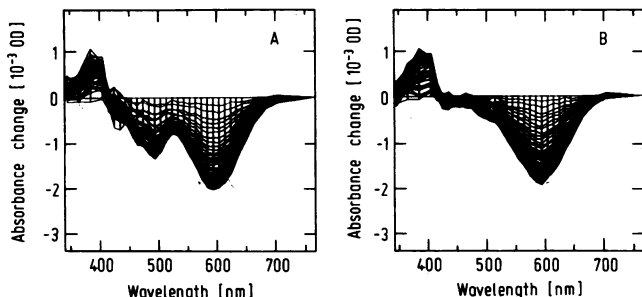


FIG. 4. Difference spectra of cell membrane fractions from cells after 24 h of growth before (A) and after (B) being washed with Tween 20 as described previously (13).

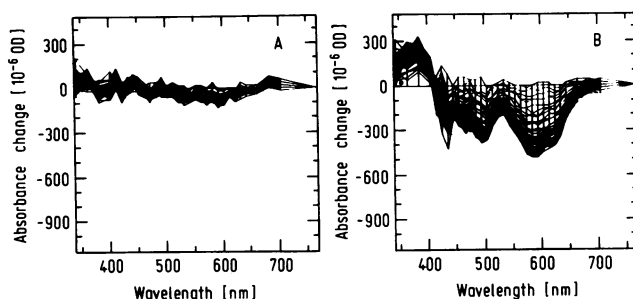


FIG. 5. Reconstitution of P₄₈₀ and SR. Difference spectra of membrane fractions from cells grown in the presence of 2 mM nicotine for 24 h before (A) and after (B) incubation with all-*trans* retinal.

absorption from cell membranes isolated from cells harvested after 24 h. Selectively, P₄₈₀ is solubilized or bleached by the Tween 20 treatment, as seen by comparison of untreated (Fig. 4A) and Tween-washed (Fig. 4B) membranes.

The reconstitution of both receptors with retinal is shown in Fig. 5. Cells were first grown in 2 mM nicotine and were harvested after 24 h. The cell membranes isolated from these cells showed no difference spectrum specific for P₄₈₀ or SR upon flash excitation (Fig. 5A). Incubation with all-*trans* retinal (final concentration, 450 nM) for 1 h in the dark apparently reconstituted both photoreceptors, as seen by the flash-induced difference spectra in Fig. 5B.

The results for photoreceptor concentrations obtained by spectroscopy were confirmed with a different approach. Figure 6 schematizes the two types of stimulus-response curves for photophobic responses in halobacteria. At photon exposures (*F*) in which all cells received more than one effective photon, 100% of the responses fall between the response time *t*_{min} and *t*_{max}; i.e., the photon exposure determined the velocity of the response as predicted by the equation given above. At photon exposures in which not all cells received an effective photon, part of the cells (or part of the events analyzed for the same cell upon repeated exposure) will react (occur) around the response time *t*_{max} of about 3 s and another part around *t*_s, the spontaneous reversal time of about 30 s (7). The fraction of cells responding to the flash in that range is a function of the flash intensity. Here photon exposure determines the probability of the occurrence of a response and stimulus-response curves that obey the Poisson statistic are obtained by plotting the frequency of response (*R*) against the log of photon exposure (*F*). In Fig. 7, results of experiments with low photon exposure are presented. Cells harvested at 38 (Fig. 7A), 64 (Fig. 7B), and 86 (Fig. 7C) h are compared for

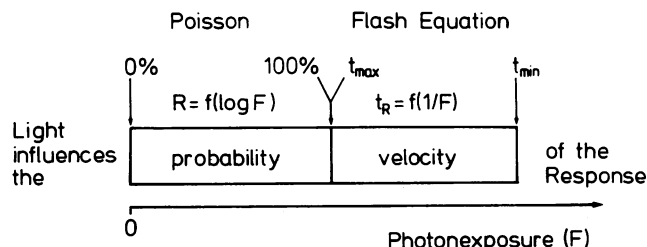


FIG. 6. Scheme for the application of stimulus-response curves to the photophobic response in *H. halobium* covering the stochastic and kinetic range as described in the text.

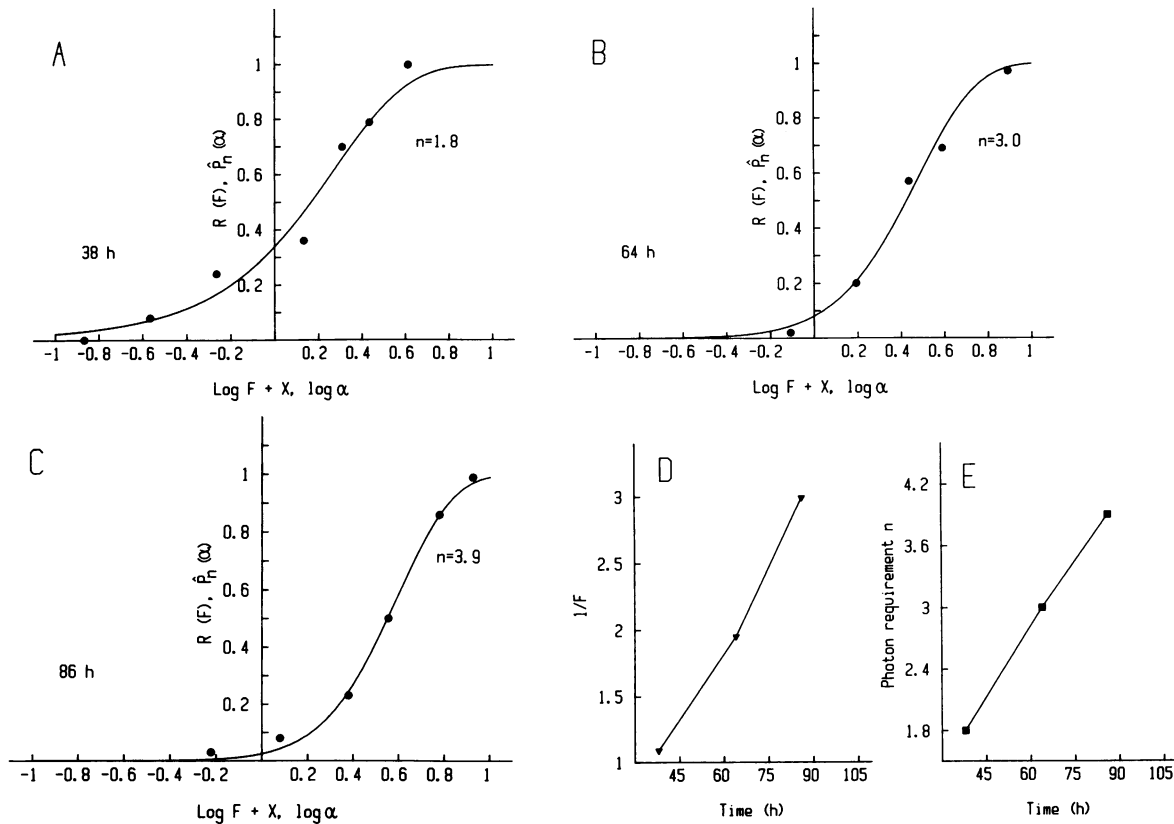


FIG. 7. Photon requirement of the SR-dependent photophobic response in halobacteria at various stages of growth. (A to C) Cells of strain Flx37, grown in a 100-ml Erlenmeyer flask, were exposed to constant orange background light (72 W/m^2) and were stimulated with flashes of near-UV light (366 nm). For each datum point, 60 to 90 cells were used and the averaged result was fitted to theoretical Poisson distribution curves by the least-square method. (For details see references 3 and 6). The x value is different in each plot. (D) The inverse value of the photon exposure (F) necessary for absorption of one efficient photon as a function of cell growth. The value $1/F$ is proportional to the number of active photoreceptor molecules in the cell. A value of 1 for F equals $1.4 \cdot 10^{-5} \text{ mol/m}^2$. (E) Minimal number of photons necessary to elicit a photophobic response as a function of cell growth. The data for panels D and E are derived from those in panels A to C. Note that the time scale in this figure may be shifted compared with that in Fig. 1 and 3, since kinetics of the cell growth in a fermenter is different from that of a shaker culture.

their SR activities. The data were fitted to Poisson distribution curves and were evaluated for the minimal photon requirement n to cause a photophobic response. In a recent paper (6), measurements of the minimal photon requirement for P_{480} -mediated photophobic response and the fit of experimental data to theoretical Poisson distribution curves (3, 6) were reported. The probability ($P_n[\alpha]$; see reference 3) is plotted versus $\log \alpha$, which reflects two components. The component to be varied in the experiments is \log photon exposure ($\log F$) and the second component, adjusted by the fitting routine, is $x = \log(R \sigma \phi \cdot \text{constant})$, where R is the photoreceptor number, σ is the cross section of absorption, and ϕ is the quantum yield. By modifying the algorithm used, it is possible to calculate theoretical curves also for decimal numbers of n , as shown in Fig. 7. Clearly the photon requirement n increases in time from 1.8 to 3.9, depending on the age of the cells (Fig. 7E). The minimal average number of photons that have to be integrated by the signal transduction chain of the cell is independent of photoreceptor concentration (see reference 3). Therefore, the cells used in our experiments already at the age of 38 h needed more than one effective photon (1.8 on average) to cause a stop response of the flagellar motor. In other words, the signal transduction chain is not maximally efficient. From the intersection of the curves with the ordinate (Fig. 7A to C),

where an average of 1 photon acts on the signal transduction chain, a measure of the relative photoreceptor concentration was obtained and plotted (Fig. 7D). This is an independent confirmation of the spectroscopic determination of SR increase during growth. We take all these results together as evidence that the decreasing photophobic response activity in aging halobacterial cells is caused by a less effective signal chain and not by a decreasing effective photoreceptor concentration.

A recent investigation revealed 1 photon to be sufficient to trigger a photophobic response by action through P_{480} in cells of a 48-h culture (6). The interpolated value for photon requirement of the SR-dependent signal chain is more than 2 in a culture of this age (Fig. 7E). This immediately suggests that the efficiencies and, therefore, the molecular compositions of the P_{480} - and the SR-dependent signal pathways are different before they merge into a common chain.

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