Bacteriorhodopsin is involved in halobacterial photoreception

(phototaxis / ΔµH⁺-receptor / Halobacterium salinarium / Halobacterium halobium)

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Communicated by Walther Stoeckenius, June 1, 1993

ABSTRACT The bacterio-opsin gene was introduced into a "blind" Halobacterium salinarium mutant that (i) lacked all the four retinal proteins [bacteriorhodopsin (BR), halorhodopsin, and sensory rhodopsins (SRs) I and II] and the transducer protein for SRI and (ii) showed neither attractant response to long wavelength light nor repellent response to short wavelength light. The resulting transformed cells acquired the capability to sense light stimuli. The cells accumulated in a light spot, demonstrating the BR-mediated orientation in spatial light gradients. As in wild-type cells, a decrease in the intensity of long wavelength light caused a repellent response by inducing reversals of swimming direction, but, in contrast to wild-type cells, a decrease in the intensity of short wavelength light also repelled the cells. An increase in light intensity evoked an attractant response (i.e., a transient suppression of spontaneous reversals). Signal processing times and adaptation kinetics were similar to the SRI-mediated reactions. However, compared to SR-mediated photoresponses, higher light intensities were necessary to induce the BR-mediated responses. The light sensitivity of the transformant was increased by adding 1 mM cyanide and decreased by the addition of arginine, agents that respectively reduce and increase the light-independent generation of the electrochemical potential difference of H⁺ ions (ΔµH⁺). A decrease in irradiance to an intensity that was still high enough to saturate BR-initiated ΔµH⁺ changes failed to induce the repellent effect, but the addition of a protonophorous uncoupler (Asp-96) sensitized the cell to these light stimuli. The BR D96N mutant (Asp-96 is replaced by Asn) with decreased proton pump activity showed strongly reduced BR-mediated responses. Azide, which increases this mutant's H⁺ pump efficiency, increased the photosensitivity of the mutant cells. Moreover, azide diminished (i) the membrane potential decreasing and (ii) repellent effects of blue light added to the orange background illumination in this mutant. We conclude that the BR-mediated photoreception is due to the light-dependent generation of ΔµH⁺. Our data are consistent with the assumption that the H. salinarium cell monitors the membrane energization level with a "protometer" system measuring total ΔµH⁺ changes or its electric potential difference component.

Halobacterium salinarium [formerly Halobacterium halo- bium (1)] contains four retinal proteins: bacteriorhodopsin (BR) (2, 3), halorhodopsin (HR) (4, 5), and sensory rhodopsins (SRs) I and II (6, 7). BR operates as a light-driven H⁺ pump (2), HR operates as a chloride pump (5), and SRI (6) and SRII (7, 8) are specialized light sensors. All four proteins undergo similar cyclic photo reactions. In BR the photocycle intermediate M, which absorbs maximally at 412 nm, arises in microseconds and decays in milliseconds (for review, see ref. 9). It transfers the Schiff base proton via the acceptor Asp-85 to the outside and accepts the proton from the cytoplasm via Asp-96. SRs are present in the same cells in much smaller amounts than BR. SRI mediates the attractant effect of the orange and red light, whereas the repellent action of shorter wavelength light is mediated by SRII and the 373-nm intermediate of the SRI photocycle (10). With the discovery of the SRs, operating as highly sensitive photon counters (11), the idea that BR and HR function as photosensors for photobehavioral responses (12-17) seemed to be rather improbable. However, a high sensitivity of SR is disadvantageous under bright light, to which the bacteria are often exposed in their natural environment, due to fast saturation of the sensory system.

Our group found that the respiratory chain and ATPase inhibitors, cyanide and dicyclohexylcarbodi-imide (DCCD), respectively, strongly increased the sensitivity of H. salinarium cells to a decrease in the intensity of bright 540- to 650-nm light. The sensitivity to blue light was not affected by these agents (12, 13). This decrease in light intensity also caused a decrease in the electrical potential difference (ΔΨ) across the bacterial membrane (14), and the magnitude of this effect was increased by cyanide and DCCD. Substituting K⁺ for Na⁺ in the incubation mixture also caused sensitization of both the repellent effect and the ΔΨ change. Addition of the respiratory substrates resulted in some desensitization of both systems.

To explain these observations, it was suggested that bacteria possess a special device to measure the electrochemical H⁺ potential difference across their membrane (ΔµH⁺) (12, 13, 15). According to the hypothesis, the ΔµH⁺ receptor called a "protometer" produces an attractant signal when ΔµH⁺ increases and a repellent signal when it decreases. In the light, BR generates ΔµH⁺, an effect that is sensed by the cell as an attractant stimulus. A decrease in the light level lowers ΔµH⁺ and, hence, causes the repellent effect. The latter is especially strong when light is the only energy source because respiration and H⁺-ATPase are inhibited by cyanide and DCCD. A second effect of DCCD operates through inhibition of the Na⁺/H⁺-antiporter, which buffers ΔµH⁺. Buffing is also absent when extracellular Na⁺ is replaced by K⁺ (18). The protometer hypothesis has been supported by several pieces of indirect evidence. (i) In H. salinarium protonophorous uncouplers decreased ΔµH⁺ and caused a repellent effect (13); (ii) cyanide and DCCD had no effect on the photoresponse of a mutant possessing SRs but lacking BR and using the K⁺ gradient as an energy source (13); and (iii) in a similar mutant, photosensing was independent of ΔΨ (19). It was difficult to exclude, however, that ΔµH⁺ somehow modulates processing of the signal produced by SRI (19, 20).

The fact that a BR-mediated photobehavioral response does exist in halobacteria was qualitatively described recently (21, 22). We now present a quantitative analysis of behavior in H.

Abbreviations: ΔµH⁺, electrochemical potential difference of H⁺ ions; ΔΨ, electric potential difference; BR, bacteriorhodopsin; HR, halorhodopsin; SR, sensory rhodopsin; DCCD, dicyclohexylcarbodi-imide; CCCP, carbonylcyanide m-chlorophenylhydrazone.

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salinarium} transformants, obtained by introducing the native or mutant bacterio-opsin genes into the Pho81 strain, which lacks all four retinal proteins. The following data demonstrate the crucial role of the BR-mediated H+ pumping in the photobehavior of these transformants.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cells.** Motile cells of \( H. \) \( \text{salinarium} \) strains Pho81, Flx3, and Flx15 (23) and Pho81-B4 and Pho81-D96N (described here) were selected on swarm plates (24) with 0.3% agar. The semisolid medium for the Pho81-B4 strain contained 20 \( \mu \)M mevinolin. Cells were taken from the visible border of the chemotactic ring in a semisolid agar plate and added to 35 ml of growth medium (2) in 100-ml Erlenmeyer flasks. Bacteria were grown at 37°C on a rotary shaker at 100 rpm and harvested during the early stationary growth phase at a density of \( \approx 2 \times 10^8 \) cells per ml. For starvation, the cells were centrifuged at 3500 \( \times g \) for 10 min, and supernatant (starvation medium) was used to dilute the cell suspension prior to microscopic analysis. Alternatively, they were washed once with NaCl buffer (4.7 M NaCl/27 mM KCl/81 mM MgSO\(_4\)/8.4 mM sodium citrate/25 mM Mops, pH 7.0) and resuspended in an appropriate medium before the start of an experiment.

**Recording of Behavioral Responses.** Two different methods were used for analyzing motility and behavior:

(i) For brief observation of motility and behavior (Figs. 5 and 6), a drop of cell suspension was placed on a slide and covered with a coverslip, and the specimen was sealed by Apiezon-Fett M to avoid evaporation and diffusion of oxygen into the cell suspension. Cell responses were evaluated by visual inspection at room temperature. For each point, up to 75 cells were tracked. The value of the repellent response was estimated as the probability for a cell to stop and swim in an opposite direction after the decrease in light intensity. The average time of response and the percentage of responsive cells were corrected for spontaneous reversals. The percentage of cells responding was plotted against the time interval between the illumination decrease and the moment when the observed cell stopped. Each cell was observed for 20 s. Since some cells did not stop during this period, the integral sum of the histograms was always \(<100\%\). For measurements of the effect of different wavelength light stimuli on the reversal frequency in Pho81 and Pho81-B4 mutants, the light stimulus was a 40% decrease in the background intensity. The light source was a mercury lamp HBO 200W (Osram). The initial light intensity was adjusted to 300 W/m\(^2\). Optical glass filters having transmittance cutoffs below 600 nm for red light, below 540 nm for orange light, and below 350 nm and above 400 nm for blue light were used.

(ii) Data for Figs. 1–4 were obtained using the motion analysis system (24). The cell suspension was applied to a slide kept at 40°C on a thermostated table, and the coverslip was sealed with petroleum jelly. Orange light from a mercury lamp passed through an interference filter (\( \lambda = 570 \pm 10 \) nm) and dichromatic mirror to a condenser of the Leitz microscope. Blue light from the xenon lamp passing an interference filter (\( \lambda = 370 \pm 10 \) nm) was applied through the objective. Two shutter systems and an automatic device for changing neutral filters were electrically synchronized with the motion analysis system. A background IR light was used for visualisation of the objects. An IR-sensitive videocamera (NEC) was connected to the Motion Analysis frame grabber (Motion Analysis, Santa Rosa, CA), and the program EXPERTVISION was used for collecting raw video data with a frequency of one frame per 100 ms. The analysis of video data and the calculation of the swimming speed and percentage of reversing cells have been described (24).

**Photoelectric Measurements.** Purple membrane sheets, containing BR and its site-specifically mutated versions, were introduced into one of the compartments of a Teflon cell. The compartments were separated by a Teflon wall with a 1-mm hole. A collodion film impregnated with an asolectin solution in decane was inserted between the two compartments, and photovoltage data were obtained as described elsewhere (25, 26).

**RESULTS AND DISCUSSION**

**Mutant Selection.** Strain Pho81 of \( H. \) \( \text{salinarium} \), which lacks all the known retinal-containing proteins and SRI transducer, was used for transformation. Mutant Pho81 is motile and fully chemotactic but does not respond to light stimuli. The mutation is pleiotropic, and the cells are incapable of synthesizing both SRs and the methyl-accepting protein possibly involved in the process of signal transduction from SRI to the flagellar motor (27, 28). A transformation system for halobacteria was initially described for \( \text{Halobacterium volcanii} \) (29) and modified later (30) for \( H. \) \( \text{salinarium} \). Plasmid vector p319 (30) contains the \( \text{bop, brp,} \) and \( \text{bat gene locus from Halobacterium sp. GRB} \). After transformation with p319, purple colonies containing BR were isolated and checked, and strain Pho81-B4 was selected for further experiments.

**Photobehavior.** Pho81-B4 cells were motile when maintained on mevinolin-containing semisolid 0.3% peptone agar. The motility of the cells of both strains decreased within 30 min when transformant cells (BR\(^+\) SRI\(^-\) SRII\(^-\)) and cells of the strain Flx15 (BR\(^-\) SRI\(^+\) SRII\(^-\)); parent for Pho81) were diluted with starvation medium without arginine and the samples were sealed to prevent the evaporation and to achieve oxygen limitation. The decrease in motility rate was less pronounced in the BR\(^+\) transformant Pho81-B4. This was obviously due to a generation of \( \Delta \mu_H \) in the light by BR (Fig. 1A).

As shown in Fig. 1B, not only a SR-containing strain but also the BR-containing transformant accumulated in a light spot under the microscope. When supplied with arginine,
cells of the Pho81 strain did not accumulate (data not shown). The quantity of the motile Flx15 cells in a spot decreased after 30 min, with some of the cells moving away from the spot either passively, due to Brownian motion, or actively, due to an inhibition of the reversal frequency when the level of $\Delta \mu_H$ fell below a certain limit.

Light stimuli—i.e., a decrease or an increase in the orange light intensity—provoked a behavioral response in the transformed cells (Fig. 2). A 90% decrease of orange light intensity induced the reversal of the cells, whereas a 90% increase of intensity suppressed spontaneous reversals for $\approx 20$ s. The maximum number of reversals was observed 1.5 s after the stimulus; this delay proved to be similar to the excitation lag-phase time for the SRI-mediated photoresponse (compare A and C in Fig. 2). Approximately 6 sec after the repellent stimuli (i.e., a decrease in the intensity of the orange light), the reversal frequency reached its prestimulus level. However, adaptation to an attractant stimulus (i.e., to an increase in the intensity of orange light) required more time (20 sec) than adaptation to a repellent stimulus in both SRI and BR-containing cells. As expected, the parental Pho81 cells did not respond to the changes in the light intensity at any wavelength studied (data not shown).

A decrease in the intensity of 350- to 400-nm light caused a repellent response of the Pho81-B4 cells (21). Thus, BR seems to restore the photosensing ability but with the difference that a decrease in blue light intensity, like that of orange and red light, is a repellent stimulus for Pho81-B4 cells. It is noteworthy that the blue light quanta are capable of stimulating the proton pump activity of the BR, which seems to function as a broad band light intensity receptor in halobacterial cell (13).

Pulse stimuli with a duration time as short as 0.2 s (compare with the average response time of the cells, which is 1.5 s), still induced a 50% photoresponse behavior (Fig. 3). The cellular responses were initiated at a time when $A^-$ has already regained its prestimulus level (25, 31). Therefore, it seems unlikely that the membrane potential change directly activates the switch complex of the motor. The signal-processing times in the range of seconds are similar to those of the SR-dependent pathways. Nevertheless BR and SR must use different transducing proteins at the initial step of signal processing because the Pho81-B4 strain lacks the methylaccepting transducer components of the SRI and SRII pathways (27).

Fig. 4 shows the fluence–response curves for the BR- and SR-containing strains. As a stimulus, we used switching off the orange light ($\lambda = 572 \pm 10$ nm) at various initial light intensities. Fig. 4A shows that the BR-mediated response requires light of higher intensity than the SRI-mediated response. Addition of an uncoupler of oxidative phosphorylation, carbonylcyanide m-chlorophenylhydrazone (CCCP), shifted the curve recorded with Pho81-B4 cells to higher intensities.

The swimming speed of halobacterial cells may be used as a relative measure of $\Delta \mu_H$, since bacterial flagella are driven by proton-motive force. By comparing Fig. 4 A and B, one can see that saturation of the sensory system in the transformant Pho81-B4 occurred when the swimming speed reached half of the maximal value. This may be due to the fact that the BR-dependent system is sensitive to changes of the $\Delta \mu_H$ value in time [$\Delta (\Delta \mu_H)/\Delta t$], whereas the flagellar motility depends on the steady-state level of $\Delta \mu_H$.

As shown in Fig. 5, 1 mM cyanide markedly increased the photosensitivity of the cells. It was also found that a higher cyanide concentration (5 mM) strongly inhibited this photoresponse. Quantitative evaluation revealed that for strain Flx3 all the cyanide concentrations used were slightly inhibi-
accumulation of BR and AP of the blue mediate cells decrease azide photobehavior mediated (35), also intermediate (26, 33, 34). on produce ATP. Subsequent substitution generation of corresponds to all the cells be

Fig. 5. Effect of the potassium cyanide on the reversal frequency in Pho81-B4 and Flx3 strains. The light stimulus was a 40% decrease in the illumination (572 ± 10 nm). Each bar indicates the percentage of cells that responded at a defined time interval. Here 100% corresponds to all the cells that reversed during the time of observation (1 min).

Fig. 6. Photoresponses of transformed cells containing the mutated (D96N) bacterio-opsin gene. (A and B) Ninety percent decrease in orange light intensity. (C and D) Ninety percent increase in blue light intensity with an orange background light. (B) Five millimolar sodium azide was added. (D) Two and five-tenths millimolar sodium azide was added. Orange light (572 ± 10 nm; initial intensity of 1.6 \times 10^{14} \text{ quanta per mm}^2 \text{ per s}) and blue light (450 ± 20 nm; initial intensity 3.67 \times 10^{13} \text{ quanta per mm}^2 \text{ per s}) were applied through the microscope condensor and objective, respectively.

orange background illumination to the D96N mutant should decrease ΔΨ and, hence, induce the repellent response with both effects abolished by azide. The experiment clearly proved these relationships. The blue light, which had an attractant effect on the Pho81-B4 strain in the absence of orange background light, turned out to be a repellent in its presence. Such an effect was much more pronounced in the Pho81-D96N mutant due to a higher stationary concentration of a blue light-absorbing intermediate M_{412}.

Photoelectric Responses. Fig. 7 demonstrates the photoelectic responses of BR-containing membrane fragments ("purple sheets") adsorbed on the surface of the collodion film impregnated with decane solution of phospholipids. Continuous orange illumination of the D96N mutant purple membranes resulted in the generation of a ΔΨ (Fig. 7A) that was strongly enhanced by azide (Fig. 7C). Applying blue light after orange caused a small but measurable ΔΨ decrease (Fig.

Fig. 7. Electrical potential generation by the purple membrane sheets adsorbed on a collodion film. (A–C) BR D96N mutant. (D) Native BR. In C, 2.5 mM NaN3 was added.
Such a decrease was absent when azide was present (Fig. 7C). When the orange light was switched off, the $\Delta \psi$ level decreased at a faster rate but was incomplete in the presence of blue light (compare A and B in Fig. 7). In the wild-type purple membranes, the orange light produced high $\Delta \psi$, which was not decreased by blue light, even in the sample without azide (Fig. 7D). The orange light intensity in Fig. 7 B and D was the same and low enough to prevent any measurable accumulation of the M intermediate in the wild-type purple membranes but not in those containing D96N BR. Therefore, the blue light effect on $\Delta \psi$ (33) is seen only in the mutant.

Conclusions. In principle, any molecule that would be able to (i) sense $\Delta \mu_H^-$ or its constituents and (ii) interact with the components of the signal transduction chain may be called a protomter, no matter what its additional function might be in the cell. The data presented here allow us to set some limits for the proposed $\Delta \mu_H^-$ sensor:

(i) A direct effect of the membrane potential on the components of the switch complex of the flagellar motor is unlikely because the response time of the cells is much longer than the time required to set a new $\Delta \psi$ steady state. The inability of the switch mutant transformed with the bacterioopsin gene to respond to light stimuli (21) is in accordance with this conclusion.

(ii) Cytoplasmic pH and periplasmic pH are known to be sensed by some eubacteria (38, 39). These parameters are related to the $\Delta \psi$ component of $\Delta \mu_H^-$. However, the pH changes seem to be too slow to cause the observed photochemical responses. Any fast $\Delta \mu_H^-$ changes in halobacteria are most probably due to the $\Delta \psi$, not $\Delta \psi$ changes. Thus the *H. salinarium* protomter may, in principle, be a $\Delta \psi$ sensor.

(iii) The information transfer pathway from BR to the flagellar motor does not seem to include the methyl-accepting protein(s) involved in SR-mediated photosensing (21, 27, 28). However, one may not exclude the possibility that SRI (20), when present, contributes to the $\Delta \mu_H^-$ sensing since its photocycling rate depends on the $\Delta \psi$ level.

(iv) The $\Delta \mu_H^-$-dependent pathway was inhibited by high concentrations of cyanide at which the SR system still functioned, at least in the Flx3 strain.

SRs are apparently responsible for photoreception at low light intensities, including color discrimination, whereas BR is involved in the photoreception at high light intensities, when the SR-mediated photoattractant system is saturated. Both situations are characteristic of the organism's natural environment.

Prof. J. L. Spudich is acknowledged for providing halobacterial mutant strain Pbr01. We thank Prof. W. Stoeckenius, Prof. E. Banmerg, Dr. J. Schiozawa, and Dr. W. Havelka for critical comments and discussion. This study was supported by research grants from the Moscow State University, Russian State Program "Frontiers in Genetcs", and Max-Planck-Gesellschaft.