BACTERIORHODOPSIN INDUCES A LIGHT-SCATTERING CHANGE IN *HALOBACTERIUM HALOBIUM*

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ABSTRACT

When suspensions of *Halobacterium halobium* are exposed to bright light, the light-scattering properties of the bacteria change. This light-scattering response can produce a transmission decrease of about 1% throughout the red and near-infrared region. The action spectrum for the light-scattering response appropriately matches the absorption spectrum of bacteriorhodopsin. The response is eliminated by cyanide p-trifluoro-methoxyphenylhydrazone, a proton ionophore, and by triphenylmethylphosphonium, a membrane permanent cation. A mild hypertonic shock induces a similar light-scattering change, suggesting that bright light causes the bacteria to shrink about 1% in volume, thereby producing the light-scattering response.

KEY WORDS *Halobacterium halobium* · bacteriorhodopsin · light-scattering · bioenergetics

Halobacterium halobium, when exposed to bright light and low O_2, synthesizes bacteriorhodopsin which aggregates to form distinct patches in the plasma membrane, the "purple membrane" (10). Bacteriorhodopsin has been shown to function as a light-driven proton pump (11). The proton gradient produced by bacteriorhodopsin is directly responsible for amino acid accumulation (6), Na^+ movements (5), and ATP synthesis (2).

Energy transduction in mitochondria and chloroplasts also involves proton translocations, and during energy transduction these organelles undergo changes in volume and structure that alter their light-scattering properties. We report here that the light-scattering properties of *Halobacterium halobium* also change during photoenergy transduction.

MATERIALS AND METHODS

Halobacterium halobium strains R_1 and S_9 were used in these experiments. The cells were grown at 38°C on a gyratory shaker under continuous illumination. The growth medium consisted of oxoid peptone 1% wt/vol in a basal salt solution containing 4.2 M NaCl, 0.03 M KCl, 0.08 M $MgSO_4$ and 0.002 M $CaCl_2$ at pH 7.4. The bacteria were grown to a cell density of about 10^9 cells/cm^3 and a bacteriorhodopsin content of about 10^{-14} g/cell. Bacteriorhodopsin content was determined by the method of Danon and Stoeckenius (2). The bacteria were then stored in their growth medium at room temperature under continuous illumination from a 75-W incandescent desk lamp approximately 10 inches from the storage flask. The bacteria can be stored for days in this manner with little loss in the ability to reproduce the results described below. Cells were harvested for the experiments by centrifugation and then resuspended in a basal salt solution. The pH of such basal salt suspensions of freshly harvested bacteria was 7.8 to 8.0. All experiments were done at room temperature and in this pH range, unless otherwise indicated.

Changes in the transmission of the cell suspensions were measured in a Shimadzu MPS-50L microspectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan) (MSP) or in a Cary 17 spectrophotometer (Cary Assay Burners, BicoBraun International, Burbank, Calif.). For experiments with the Cary 17, the bacteria were suspended in a 1-cm pathlength microcuvette with

a concentration of 5×10^8 cells/cm^3; for MSP experiments they were suspended in a microcell with a path length of 350 μm at a concentration of 10^{10} cells/cm^3. In both cases, the cell concentration was adjusted to yield an optical density of about 0.8 at 1,000 nm. The actinic-light source was a 20-W quartz iodine lamp. To ensure relatively uniform actinic illumination throughout the suspension of bacteria in the Cary 17, the sample was illuminated from the side of the microcuvette, thereby reducing the actinic-light path to 3 mm and the effective optical density of the sample to about 0.6 for the 580 nm actinic light. The heat of the actinic lamp was reduced by using Schott & Gen. KG-3 and BG-38 infrared blocking filters (Schott Optical Glass Inc., Duryea, Pa.), a 582 ± 17 nm interference filter, and a 3-cm-thick flat-sided flask filled with water. To observe the action spectrum, relatively monochromatic actinic light was obtained by replacing the 582 ± 17 nm interference filter with Oriel narrow band interference filters (Oriel Corp. of America, Stamford, Conn.) with band widths of 6 nm. Actinic light intensities were measured with a calibrated SGD-100 photodiode from EG & G, Inc. (Electro-Optics Div., Salem, Mass.). To prevent the actinic light from reaching the photomultiplier, a Ditric 780 nm cut-on filter (Ditric Optics, Inc., Marlboro, Mass.) was placed in front of the light entrance to the photomultiplier.

Most experiments were done with aerobic suspensions of bacteria. In the Cary 17 experiments the suspensions of bacteria were in either an open cuvette or a microcuvette exposed to the atmosphere. Anaerobic conditions were produced in some experiments by bubbling nitrogen gas through a cell suspension for 15 min, then quickly placing the suspension in the Cary 17 sample chamber which was continuously purged with nitrogen for a few hours before and during the experiments.

Changes in the pH of aerobic suspensions of bacteria during illumination were measured with a Beckman combination glass electrode (Beckman Instruments no. 39505, Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) and a Corning 110 pH meter (Corning Glass Works, Science Products Div., Corning, N. Y.). The output from the pH meter was recorded by an Omni Scribe strip chart recorder (Houston Instruments Div., Bausch & Lomb, Inc., Austin, Tex.). The suspension was continuously stirred in a 1-cm pathlength cuvette with a small Teflon-coated magnetic stirring bar (Cole-Parmer Instrument Co., Chicago, Ill.). The half-time response of this system to a pH pulse was approximately 30 s. The pH of the suspensions of bacteria was adjusted with NaOH or HCl solutions. The illumination of the cuvette was identical to the illumination described above. Cell concentrations for the pH experiments were about 5×10^8 cells/cm^3.

RESULTS AND DISCUSSION

Illumination of an aerobic suspension of *Halobacterium halobium* typically produced a transmission decrease which returned to the original base line when the actinic light was turned off (Fig. 1). There were brief latent periods before both the rise and decay phases of the transmission change, i.e., the transmission did not change during the first few seconds after the actinic light was turned on or off. Both the rise and decay half-times for any particular response were about the same. (The half-times were measured from the beginning of the rise or decay phases.) Our most intense actinic light produced maximum transmission changes of between 1 and 2%. Fig. 2 shows the amplitude and rise-time for the transmission change as a function of actinic light intensity for a typical suspension of bacteria. Bacterial suspensions from different culture flasks yielded some variability in the rise-time and amplitude of the transmission change for a given actinic-light intensity. The rise and decay half-times ranged from 10 to 30 s. However, in every sample the amplitude of the transmission change increased with actinic light intensity until it saturated at a maximum amplitude of 1–2%. In contrast, the rise times of the responses were almost constant, independent of actinic-light intensity.

FIGURE 1 Transmission decrease produced by illuminating an aerobic suspension of *Halobacterium halobium* S_9 as recorded in a Cary 17. Measuring wavelength 1,000 nm; actinic wavelength 582 ± 17 nm; actinic intensity 6×10^{15} photons/cm^2 s; pH 7.8.

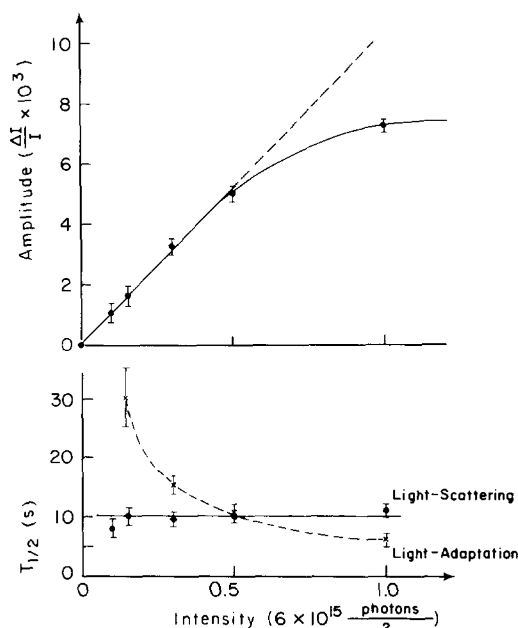

FIGURE 2 Amplitude and rise time of the light-scattering response, and half-time for light adaptation of bacteriorhodopsin, as functions of actinic-light intensity. All observations were made on aerobic suspensions of *Halobacterium halobium* S_9 with the Cary 17. Measuring wavelengths: light-scattering response, 1,000 nm; light adaptation of bacteriorhodopsin, 575 nm; actinic wavelength, 582 ± 17 nm; pH 7.8. To avoid "blinding" the photomultiplier, changes of absorbance at 575 nm were recorded between successive exposures to the actinic light, and the half-time shown is for total actinic exposure time. Error bars indicate the range of values found in several recordings from the same bacterial suspensions.

The transmission change produced by illumination of an aerobic suspension of bacteria was similar to light-induced transmission change produced by the same suspension under anaerobic conditions. 6 mM KCN, an electron transport inhibitor, had little effect on the light-induced transmission change. Thus this light response is basically similar under aerobic and anaerobic condition.

We conclude from the following evidence that the transmission change is produced by a change in the light-scattering properties of the bacteria: (*a*) The amplitude of the transmission change depends strongly on the numerical aperture of the collecting objective in the MSP experiments: when the numerical aperture of the incident-measuring light was set at 0.08 and the numerical aperture of the collecting objective was decreased from 0.2 to 0.004, the amplitude of the observed transmission change increased many fold. (*b*) The intensity of the light scattered off the optic axis increased during illumination of the bacterial suspension: when an annulus, whose inside and outside "numerical apertures" were 0.2 and 0.4, was placed in the collecting objective, and the incident light had a numerical aperture of 0.08, the amount of light reaching the photomultiplier increased during illumination of the bacteria, producing an apparent increase in transmission. (*c*) The transmission change depended only weakly on the wavelength of the measuring light: the amplitude of the transmission change increased less than 20% when the measuring light was varied from 1,300 to 650 nm. It seems highly unlikely that a change in some pigment could produce such a flat difference spectrum over this range of measuring wavelengths. Also, there are no known pigments in *Halobacterium halobium* which absorb strongly in 750–1,300 nm wavelength range. Thus, from these results, the light-induced transmission change is best explained by a change in the light-scattering properties of the bacteria, a change which decreases the light scattered in the forward direction and increases the light scattered off the optic axis.[1]

Bacteriorhodopsin must initiate this light-induced light-scattering response since: (*a*) bacteria with less than 10^{-15} g/cell of bacteriorhodopsin did not produce a detectable light-scattering change, and (*b*), as shown in Fig. 3, the action spectrum of the light-induced response appropriately matches the absorption spectrum of light-adapted bacteriorhodopsin, after corrections are made for the extensive scattering of actinic light and absorption by screening pigments. The S_9 mutant of *Halobacterium halobium* was used to obtain the action spectrum because it contains less screening pigments than the R_1 strain. However, even with the S_9 strain, considerable correc-

[1] S. Saphon and W. Stoeckenius have observed a transmission decrease during illumination of *Halobacterium halobium* that is abolished by 10^{-5} M FCCP (personal communication). They could observe this transmission change at measuring wavelengths between 415 and 484 nm, but not at 672 nm. They found no difference in the amplitude of the transmission change as the photomultiplier was moved from very close to the sample cuvette to 60 cm away; thus this light-induced response may differ from the light-scattering change we report here.

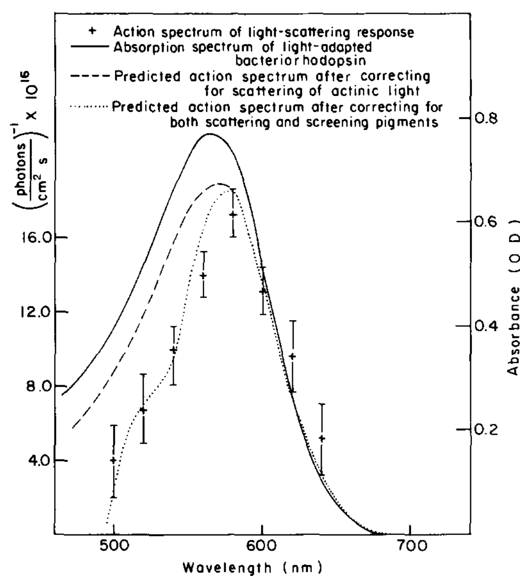

FIGURE 3 Action spectrum of the light-scattering response plotted as the reciprocal of the actinic-photon flux needed to produce a criterion response amplitude as measured at 1,000 nm. The error bars indicate the range of values found in several observations from different aerobic suspensions of bacteria. The absorption spectrum of bacteriorhodopsin was obtained from a suspension of isolated purple membrane fragments. The correction for light-scattering was obtained by recording the extinction spectrum of the sample and calculating from this the effective average intensity of the actinic light throughout the sample. The correction for screening pigments was obtained by observing the absorption spectrum of the screening pigments after lysing the cells and bleaching the bacteriorhodopsin with cetyltrimethylammonium bromide. This absorption spectrum was used to calculate the effective average intensity of the actinic light as a function of wavelength. Because the effective average intensity was reduced by scattering and screening pigments at short wavelengths, the amount of short-wavelength actinic light needed to obtain a criterion response was greater.

tions must still be made, as shown in the figure.

Because bacteriorhodopsin can exist in either a light-adapted (λ_{max} = 568 nm) or a dark-adapted (λ_{max} = 560 nm) form (10), we examined the relationship between the light-scattering response and the processes of light and dark adaptation in bacteriorhodopsin. Light adaptation could be measured with the S_9 strain by observing the increase in extinction at 575 nm that occurs during the conversion of dark-adapted bacteriorhodopsin to the light-adapted state. (The amplitude of the light-scattering change at this measuring wavelength was negligibly small due to the high degree of multiple-scattering in our suspensions at shorter measuring wavelengths.) The half-time for light-adaptation as a function of actinic-light intensity is shown in Fig. 2. We found that bacteriorhodopsin light-adapted within tens of seconds with our actinic illumination and dark-adapted with a half-time of about 1 h at room temperature. The extinction change at 575 nm was easily distinguished from the light-scattering response, because it occurred only after the first exposure to actinic light after a few hours of dark-adaptation. In contrast, numerous light-scattering responses could be easily obtained from a single suspension of bacteria by repeated illumination, without waiting for the bacteriorhodopsin to dark-adapt. Thus the bacteriorhodopsin does not have to be dark-adapted to produce the light-scattering response. Moreover, when the intensity of the actinic light was increased, the half-time for light-adaptation decreased but the half-time for the light-scattering response did not change. Hence, the two processes seem functionally independent.

The light-scattering response observed during illumination could be caused by changes in the structure, shape, or size of the bacteria, or even to phototaxic responses (3). However, we think it unlikely that the light-scattering response results from phototaxis, because the light-scattering response was not significantly changed by constant stirring of the bacterial suspension nor by mechanically deflagellating the bacteria in a blender (Omni-mixer, E. I. Dupont de Nemours Co., Instruments Products Division, Sorvall Operations, Newtown, Conn.). (The effectiveness of this deflagellation procedure was verified by electron microscopy.) We also found that isolated purple-membrane fragments did not produce a detectable light-scattering change when illuminated, making it unlikely that a structural change in the purple membrane is responsible for the light-scattering response. Thus, the simplest explanation for the light-induced light-scattering change would be a light-induced volume and(or) shape change of the bacteria.

To examine how bacterial volume affects the light-scattering properties of the bacteria, we subjected the bacteria to gentle hypotonic and hypertonic NaCl shocks and observed the resulting transmission changes. The bacteria were osmotically shocked by adding hypotonic or hyper-

tonic NaCl solutions to basal salt suspensions of bacteria. To ensure that a constant number of cells remained in the measuring beam after the osmotic shock we used a Hellma two-compartment mixing cuvette (Hellma Cells, Inc., Jamaica, N. Y.). Also, the refractive indexes of the shocking solution and bacterial suspension were carefully matched with bovine albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.) with Abbe refractometer (Bausch and Lomb Inc., Ophthalmic & Consumer Products Div., Rochester, N. Y.), so that the shock would not change the refractive index of the suspending medium. Hypertonic shocks were found to produce transmission decreases, while hypotonic shocks produced transmission increases. We found that a 1% hypertonic shock would produce a transmission decrease similar in amplitude to the maximum light-induced light-scattering change. Light-scattering changes that occur when cells shrink or swell by a gain or loss of water, i.e., no change in dry weight, have been analyzed theoretically (4, 8, 9). Koch (4) found that $\log \frac{1}{\text{transmission}}$ is approximately proportional to $V^{-2/3}$ for nonabsorbing spheres the size of bacteria, where V is the volume of the sphere. This result indicates that a 1% transmission decrease corresponds to a volume shrinkage of about 1% which accords well with the results of the osmotic shock experiments. Thus the light-induced light-scattering change could easily be the result of the bacteria shrinking in volume on the order of 1% in response to a bright light.

We examined the relationship between the light-induced proton movements and the light-induced cell-volume shrinkage by observing both the pH and light-scatter responses from the same suspension of bacteria at identical illumination. Because the light-induced pH response depends upon the initial pH of the suspension of bacteria (1), we examined both the pH and light-scattering responses at three different pH levels by using aerobic suspensions of bacteria. We found that the pH response differed markedly in sign, magnitude, and time course, depending on the initial pH. In contrast, the light-scattering response always indicated cell-volume shrinkage, regardless of the initial pH and the net proton movement. For example, at pH 7.8 the pH response consisted of a prompt alkalinization, at pH 5.2 a prompt acidification occurred, and at pH 6.5 (Fig. 4) there was an initial alkalinization followed by an acidification. However, at all of these initial pH levels, the light-scattering response consisted simply of a transmission decrease similar to that shown in Fig. 1. A direct comparison between the light-scattering response and the initial pH response from the same suspension of bacteria is shown in Fig. 4*b* for an initial pH of 6.5. Although both responses occur over the same time interval of a few minutes, they appear to have no direct functional relationship.

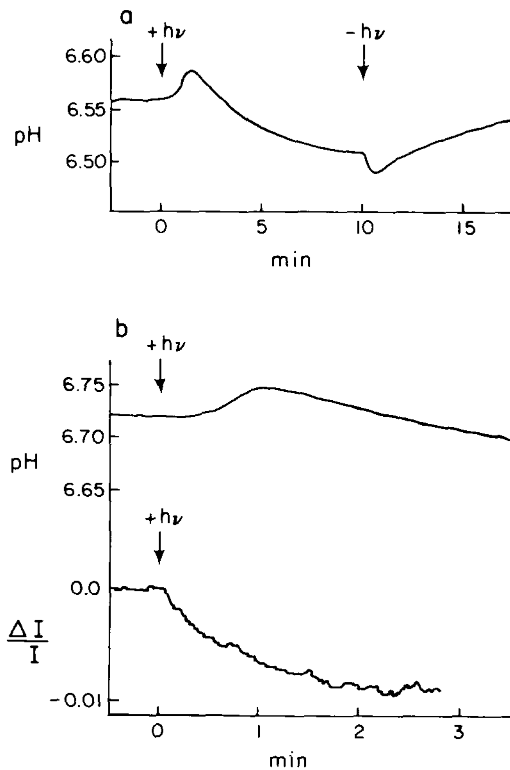

FIGURE 4 Light-induced pH and light-scattering responses from an aerobic suspension of *Halobacterium halobium* S_9. (*a*) Tracing of a typical light-induced pH response from a suspension of bacteria of approximately pH 6.5 recorded as described in Methods. Actinic intensity 6×10^{15} photons/cm^2 s; actinic wavelength 582 ± 17 nm. (*b*) Tracings of light-induced pH and light-scattering responses observed from the same suspension of bacteria. The light-induced light-scattering change was observed with the Cary 17. Measuring wavelength 1.000 nm; actinic wavelength 582 ± 17 nm; actinic intensity 6×10^{15} photons/cm^2 s.

The light-induced shrinkage in cell volume appears to depend in some way on the electrochemical potential across the plasma membrane because (*a*) 10^{-6} M of a proton ionophore, cyanide

p-trifluoro-methoxyphenylhydrazone (FCCP, E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.), diminished the light-scattering response to below detection limits (probably by reducing the proton gradient across the membrane) and (*b*) when the electrical potential across the membrane was reduced with 5 mM triphenylmethylphosphonium ($TPMP^+$, K&K Laboratories, Plainview, N. Y.), a membrane permanent cation, the light-scattering change was also reduced, to below detection limits. Thus the light-induced volume shrinkage seems to require the presence of both the proton gradient and the membrane potential.

An obvious mechanism that could produce volume shrinkage would be a net movement of osmotic particles out of the cells during illumination. Protons are actively pumped out of halobacteria during illumination (1), and a light-dependent Na^+ efflux has also been observed in *Halobacterium halobium* cell envelope vesicles (5, 7). The degree to which this electrogenic ion pumping is balanced electrically by other ion movements; e.g., K^+ and Cl^-, would determine whether the bacteria would change in volume. Because the cells shrink in volume, it seems likely that some Cl^- efflux occurs in response to the active pumping of H^+ and(or) Na^+ out of the bacteria. It is unlikely that protons alone contribute significantly to a net osmotic efflux of particles, because net proton movement varied markedly with the initial pH but the light-induced volume shrinkage did not. Thus, light-dependent Na^+ efflux seems a more probable mechanism for producing osmotic shrinkage.

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