Phototaxis and Membrane Potential in the Photosynthetic Bacterium \textit{Rhodospirillum rubrum}

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Cells of the photosynthetic bacterium \textit{Rhodospirillum rubrum} cultivated anaerobically in light show phototaxis. The behavior of individual cells in response to the phenomenon is reversal(s) of the swimming direction when the intensity of the light available to them abruptly decreases. The tactic response was inhibited by antimycin, an inhibitor of the photosynthetic electron transfer system. The inhibitory effect of antimycin was overcome by phenazine methosulfate. Motility of the cells was not impaired by antimycin under aerobic conditions. Valinomycin plus potassium also inhibited their phototactic response; however, valinomycin or potassium alone had no effect. A change in membrane potential of the cells was measured as an absorbance change of carotenoid. Changes in the membrane potential caused by "on-off" light were prevented by antimycin and by valinomycin plus potassium, but not by antimycin plus phenazine methosulfate nor valinomycin or potassium alone. The results indicated that the phototactic response of \textit{R. rubrum} is mediated by a sudden change in electron flow in the photosynthetic electron transfer system, and that the membrane potential plays an important role in manifestation of the response.

Bacterial phototaxis has been observed in photosynthetic bacteria (6, 22) and in \textit{Halobacterium halobium} (12). The photoreceptor pigments responsible for positive phototaxis (phototaxis towards light) have been identified as bacteriochlorophyll and carotenoids in photosynthetic bacteria (5, 19) and as bacteriorhodopsin in \textit{H. halobium} (12). The phototactic behavior of individual cells of photosynthetic bacteria has been extensively examined (10): the bacteria show abrupt changes in direction when they swim from a light to a dark field, and some of them return to the light field; however, they fail to show such a response when they swim from a dark to a light field. As a consequence of these responses, the number of organisms in a light spot projected onto a suspension of bacteria increases with time. The change in swimming direction can be induced by temporal decrease of an actinic light intensity impinging on them. This observation suggested that the phototactic response is the result of sensing temporal, not spatial, differences in light intensity.

In chemotaxis of \textit{Escherichia coli} and of \textit{Salmonella typhimurium}, chemoreceptor molecules of several attractants (chemicals that induce positive chemotaxis) have been identified (1, 11, 18). The sugar chemoreceptors are proteins that are constituents of active transport systems. The chemotactic behavior of individual cells of \textit{E. coli} and \textit{S. typhimurium} has also been examined (1, 3, 16, 26, 30). The bacteria modulate the frequency of change in direction when they swim up or down a spatial gradient of a chemotactic stimulus; thus, their attraction or migration from it is attained. This modulation is also observed when they are exposed to a temporal change in concentration of the stimulating chemicals.

Whereas cellular receptors of the tactic stimuli and swimming behaviors induced by the stimuli have been well documented in bacterial phototaxis, as well as in bacterial chemotaxis, how the stimuli are converted to a tactic signal that modulates the frequency of change in their swimming direction is unclear.

In this paper we show evidence that reversals of the swimming direction of the photosynthetic bacterium \textit{Rhodospirillum rubrum} induced by a sudden decrease in the actinic light intensity are mediated by a sudden decrease of electron flow in the photosynthetic electron transfer system and that membrane potential plays an important role in the response.

**MATERIALS AND METHODS**

Bacterial strains and growth of cells. Wild-type \textit{R. rubrum} (S-1) was obtained from G. Soe, and the carotenoid-less blue-green mutant (G-9) was from Y.
Kobayashi, to whom we are indebted. The bacteria were cultivated in light at 28°C in a 120-ml screw-capped bottle containing a synthetic medium whose composition was as described previously (8), except that Casamino Acids was omitted from the medium. Illumination was provided by one 300-W flood lamp (Toshiba) placed 80 cm from the culture bottle. The bacteria at the late exponential to stationary growth phase were harvested, centrifuged, washed at least two times with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6), suspended in the appropriate solution, and then used for experiments. In the experiments reported in Table 3, the bacteria were washed in 10 mM sodium phosphate buffer (pH 6.8).

Measurement of phototactic response. To examine quantitatively the phototactic response of the bacteria, an apparatus for giving successive phototactic stimuli to the bacteria was devised (Fig. 1). Two light beams, one for an actinic light and another for an observing light, were projected onto a phase-contrast microscope condenser through a half mirror. The actinic light beam from a tungsten-halogen lamp (Ushio, JC-24V-150W) was passed through a 4-cm cuvette containing water, chopped by a rotary sector for 0.5 s every 2 s, and then projected on a bacterial suspension on a glass slide through a phase-contrast condenser. The intensity of the actinic light, measured with a YSI-Kettering model 65 radiometer, was 500 W/m². The observing light beam of a 30-W tungsten lamp (Nikon) was filtered by a thermo-cut-off filter (Hoya, HA-50) and a green interference filter (Toshiba, KL-56; peak transmission at 558 nm, with a half band width of 14 nm) and then continuously projected on the bacterial suspension. The intensity of the observing light was 3 W/m² or below.

The tactic response of R. rubrum induced by a sudden decrease in actinic light intensity is simple: the bacteria reverse their swimming direction after being stimulated (6, 10). Therefore, as an index of their phototactic activity, we measured the fraction of bacteria that showed reversals of swimming direction within 0.5 s after the actinic light was turned off in repeated "on" (1.5 s) and "off" (0.5 s) periods, by direct microscopic observations made at an ambient temperature of 30°C. When more than 50 bacteria were counted, the standard deviation of the measured values from the same sample did not exceed 20% of the mean value.

Measurement of an absorbance change in bacterial suspension. Measurement of light-induced absorbance (A) change was carried out with a Hitachi 356 double-beam spectrophotometer. The wavelength used for measuring the absorbance change of carotenoid in wild-type cells (S-1) was 525 nm, with 510 nm as a reference. Those used for measuring that of cytochrome c₂ and reaction center bacteriochlorophyll in cells of the carotenoid-less mutant (G-9) were 552 and 600 nm, with 540 and 585 nm, respectively, as references. Actinic light from a tungsten-halogen lamp (Ushio, JC-24V-150W) filtered through an E-72 filter (Hoya), allowing transmission of light only above 700 nm, was projected onto a cuvette (light path, 1 cm) at a right angle to the measuring beam of the spectrophotometer. The intensity of the actinic light was 1.3 kW/m². Between the cuvette and a photomultiplier tube, two filters (Corning 9782 and HA-50) were placed to protect the photomultiplier tube from the cross-illuminating actinic light. Intermittent illumination of the actinic light (1.5 s on and 0.5 s off) was attained by insertion or removal of a sector connected to a rotary solenoid operated by an autotimer.

Estimation of cellular content of bacteriochlorophyll. Bacteriochlorophyll was extracted with acetone-methanol (7:2, vol/vol), and then the cellular content of the pigment was determined spectrophotometrically by using the absorption coefficient given by Clayton (5).

Chemicals. Valinomycin and antimycin were purchased from Boehringer Manheim. Other reagents used were of analytical grade.
RESULTS

Effect of antimycin and PMS on phototactic response and photosynthetic electron transfer in the bacteria. Cells of *R. rubrum* reverse their swimming direction when the actinic light intensity impinging onto them is abruptly decreased. Since the phototactic phenomenon, i.e., accumulation of the bacteria in a light spot projected onto a bacterial suspension, is a result of this response (6, 10), we estimated the phototactic activity of the bacteria by counting the fraction that reversed swimming direction in a 0.5-s dark period after the actinic light was turned off. The value of the fraction depended on decreased intensity of the actinic light as well as intensity of the observing light. Furthermore, sensitivity of the cells to the phototactic stimulus differed in different cultures. However, under the conditions described in Materials and Methods, 60 to 95% of the cells responded to the stimulus in control experiments.

Antimycin, a specific inhibitor of photosynthetic electron transfer (13, 33), inhibited the phototactic response of the bacteria (Table 1). Motility of the bacteria was not affected by the inhibitor under aerobic conditions. The inhibitory effect of antimycin on the phototactic response was abolished when phenazine methosulfate (PMS) was subsequently added to the bacterial suspension. PMS had a stimulative effect on phototactic response when it was added to the bacterial suspension containing no antimycin.

Antimycin affected light-induced redox changes of cytochrome c₂ measured as *A*₅₅₂⁻₅₄₀.

**Table 1. Effect of antimycin and PMS on phototactic response**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nᵃ</th>
<th>Phototaxis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (0.2%, vol/vol)</td>
<td>224</td>
<td>60ᵃ</td>
</tr>
<tr>
<td>Antimycin (20 μM)</td>
<td>129</td>
<td>5ᵈ</td>
</tr>
<tr>
<td>Ethanol (0.2%, vol/vol) + PMS</td>
<td>180</td>
<td>84⁻¹</td>
</tr>
<tr>
<td>Antimycin (20 μM) + PMS (0.2 mM)</td>
<td>163</td>
<td>67⁻¹</td>
</tr>
</tbody>
</table>

ᵃ Cells of *R. rubrum* S-1 were suspended in 10⁻² M Tris-hydrochloride buffer (pH 7.6) containing the addition.
ᵇ Number of the bacteria examined.
ᶠ Fraction of the bacteria that showed reversal(s) of swimming direction 0.5 s after actinic light was turned off.
ᵍ Phototactic response was observed 1 to 1.5 h after the addition of ethanol or antimycin.
⁻ⁱ PMS was added 1.5 h after the addition of ethanol or antimycin, and then the phototactic response was observed 10 to 20 min afterwards.

Its oxidation (decrease in *A*₅₅₂⁻₅₄₀) by light was inhibited, and its reduction after the light was turned off was prevented by the inhibitor. Redox changes of reaction center bacteriochlorophyll, measured as *A*₅₀₀⁻₅₈₅, were hardly affected by the inhibitor. Changes in carotenoid absorbance, which reflects changes in membrane potential (14, 15), were affected by the inhibitor; an increase in membrane potential (hyperpolarization) was observed after the light was first turned on, but a decrease in membrane potential (depolarization) after the light was turned off was prevented by the inhibitor. That the observed absorbance change at 525 to 510 nm corresponded to that of carotenoid, reflecting changes in membrane potential, was confirmed by the fact that, in a cell suspension of the carotenoid-less mutant (G-9), no light-induced absorbance change between the two wavelengths was observed with or without antimycin and PMS (Fig. 2).

Effect of valinomycin on phototactic response and light-induced absorbance change of carotenoid. Valinomycin is an ionophore that increases conductivity of K⁺, Rb⁺, and Cs⁺ in biological membranes; in the presence of valinomycin, these cations move down the electrochemical potential across the membrane and discharge it (9, 28). We used the ionophore to examine the role of membrane potential in the bacterial phototactic response because its effect on biological membranes was well characterized.

When valinomycin was added to the cell suspension of *R. rubrum* with or without KCl, it had no effect on the phototactic response or on the light-induced absorbance change of carotenoid. Since it is known that the outer membrane of gram-negative bacteria acts as a penetration barrier for hydrophobic antibiotics having a molecular weight of 1,100 (20), it is possible that the insensitivity of intact *R. rubrum* cells to the ionophore is due to the existence of an intact outer membrane. It was reported that ethylenediaminetetraacetate (EDTA) removed the penetration barrier (17), and thus *E. coli*, a gram-negative bacterium, became sensitive to valinomycin (27). Upon EDTA treatment, cells of *R. rubrum* also became sensitive to valinomycin: the phototactic activity of the EDTA-treated cells was strongly inhibited by valinomycin. Spontaneous reversal frequency of the cells, under steady light illumination, was also diminished by valinomycin. The concentration of EDTA required to render the cells sensitive to valinomycin differed in different cell cultures: cells in the stationary growth phase were more resistant to EDTA than those of the exponential growth phase. Cumulative results on
In the present experimental conditions, no impairment of motility was observed.
**Table 2. Effect of valinomycin on phototactic response**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Growth phase</th>
<th>EDTA concn (M)</th>
<th>Incubation time in EDTA (min)</th>
<th>Addition*</th>
<th>N°</th>
<th>Phototaxis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stationary</td>
<td>$10^{-3}$</td>
<td>42–52</td>
<td>E</td>
<td>121</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>55</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Stationary</td>
<td>$10^{-3}$</td>
<td>280–285</td>
<td>E</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>44</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Stationary</td>
<td>$10^{-3}$</td>
<td>240–245</td>
<td>E</td>
<td>64</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Late exponential</td>
<td>$10^{-4}$</td>
<td>35–50</td>
<td>E</td>
<td>51</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Late exponential</td>
<td>$10^{-4}$</td>
<td>80–83</td>
<td>E</td>
<td>61</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Stationary</td>
<td>$10^{-3}$</td>
<td>100–105</td>
<td>E</td>
<td>73</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>46</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-4}$</td>
<td>200–205</td>
<td>E</td>
<td>92</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>64</td>
<td>78</td>
</tr>
</tbody>
</table>

* V, $10^{-5}$ M valinomycin; E, 1% (vol/vol) ethanol.

* Number of bacteria examined.

![Diagram](http://jb.asm.org/)

**Fig. 3. Light-induced absorbance changes of carotenoid in EDTA-treated cells with or without the addition of valinomycin.** Light-induced absorbance changes at 525 nm, with 510 nm as a reference of the bacterial suspensions used in experiments of Table 2, were measured immediately after the measurements of phototactic response. (A) Bacterial suspension used in experiment 2 of Table 2 (bacteriochlorophyll concentration, 23 μM). (B) Bacterial suspension used in experiment 5 of Table 2 (bacteriochlorophyll concentration, 13 μM). (E) Ethanol (1%, vol/vol) added. (V) valinomycin (10 μM) added.

Comitant inhibition of bacterial phototaxis.

The effect of incubation time of the cells in EDTA solution on their phototactic response in the presence or absence of valinomycin was examined (Fig. 4). In the experiment, 10 mM CaCl₂ was added to the cell suspension to stop the action of EDTA at the indicated time. It was shown that sensitivity of the cells to valinomycin increased with increased incubation time in EDTA solution; however, prolonged incubation resulted in inhibition of phototactic response even without addition of valinomycin.

Valinomycin discharges membrane potential only when appropriate monovalent cations such as K⁺ and Rb⁺ are present. To examine whether the inhibitory effects of valinomycin are dependent on the presence of these cations, the effects of valinomycin on phototactic response and light-induced absorbance change of carotenoid in the EDTA-treated cells suspended in buffer free from these cations were examined. For this purpose, cells of *R. rubrum* S-1 at the late exponential growth phase were harvested, suspended in 10 mM sodium phosphate buffer (pH 6.8), and incubated aerobically with shaking (120 strokes per min) at 28°C for 1 h; 0.3 mM
FIG. 4. Effect of incubation time in EDTA solution on phototactic response. S-I cells harvested in the late exponential growth phase were suspended in 10^{-2} M Tris-hydrochloride buffer containing 10 mM KCl and were incubated aerobically at 28°C for 1 h. EDTA (0.1 mM) and ethanol (1%, vol/vol) or valinomycin (10 μM) were added to the bacterial suspensions, which were further incubated aerobically at 28°C. CaCl₂ (1 mM) was added to stop the action of EDTA at a time, indicated on the figure, after the addition of EDTA, and then the phototactic response was observed 15 min afterwards.

EDTA and ethanol (1%, vol/vol) or valinomycin (10 μM) were added to the cell suspension and further incubated aerobically for 30 min. CaCl₂ (2 mM) was added to stop the action of EDTA on the cells. Thirty minutes after the addition of CaCl₂, 10 mM KCl, 10 mM RbCl, or water (final concentration, 1% [vol/vol]) was added to the cell suspensions, and phototactic activities of the cells in these suspensions were measured 20 to 35 min and 40 to 55 min afterwards. The mean of two measurements is presented in Table 3. The absorbance changes of carotenoid within 0.5 s after the actinic light was turned off were measured 60 to 65 min after the addition of KCl, RbCl, or water (Table 3). When the bacteria were suspended in the 10 mM sodium phosphate buffer containing no KCl, addition of valinomycin after EDTA treatment of the cells did not affect significantly their phototactic response or the light-induced absorbance change of carotenoid. Further addition of 10 mM KCl or RbCl to the bacterial suspension inhibited the phototactic response as well as the light-induced absorbance change of carotenoid (Table 3). The results clearly indicated that the inhibitory effects of valinomycin on the phototactic response and light-induced absorbance change of carotenoid were dependent on the existence of appropriate cationic species such as K⁺ and Rb⁺, but not Na⁺.

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Monovalent cation added</th>
<th>N°</th>
<th>Phototaxis (%)</th>
<th>ΔA/μM BChl⁻ (×10⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>water (1%, vol/vol)</td>
<td>41</td>
<td>73</td>
<td>3.0</td>
</tr>
<tr>
<td>V</td>
<td>water (1%, vol/vol)</td>
<td>36</td>
<td>86</td>
<td>2.6</td>
</tr>
<tr>
<td>E</td>
<td>10⁻² M KCl</td>
<td>44</td>
<td>73</td>
<td>2.3</td>
</tr>
<tr>
<td>V</td>
<td>10⁻² M KCl</td>
<td>100</td>
<td>15</td>
<td>0.8</td>
</tr>
<tr>
<td>E</td>
<td>10⁻² M RbCl</td>
<td>41</td>
<td>80</td>
<td>2.6</td>
</tr>
<tr>
<td>V</td>
<td>10⁻² M RbCl</td>
<td>70</td>
<td>16</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* E, Ethanol (1%, vol/vol); V, valinomycin (10⁻⁵ M).
* Number of bacteria examined.
* Mean of 10 absorbance changes of carotenoid (A₅₂₅±₅₁₀) per micromolar bacteriochlorophyll (BChl) 0.5 s after the actinic light was turned off.

**DISCUSSION**

Pigmented cells of *R. rubrum* reverse their swimming direction when the intensity of light impinging on them is abruptly decreased. Since the reversal of swimming direction is a key movement in attaining phototactic accumulation (6, 10), we examined the fraction of bacteria that reverse their swimming direction after a sudden decrease in light intensity to estimate their phototactic activity. Under the conditions of the experiments, very few cells showed repeated reversals within 0.5 s after a sudden decrease in actinic light intensity; i.e., almost all of the cells showed one or no reversals after the stimulus.

Photoreceptor pigments responsible for the phototactic response of the bacteria have been identified as bacteriochlorophyll and carotenoids (4, 19). These are pigments driving photosynthesis. Thus, the hypothesis that phototaxis is induced by a transient disturbance of photosynthetic metabolism was proposed and widely accepted (6). A requirement of photosynthetic electron transfer for the manifestation of phototaxis has been claimed by Throm (32). He observed that inhibitors of photosynthetic electron transfer affected the maximum level of accumulation of cells in a light spot. However, the rate and the maximum level of bacterial accumulation in a light spot may be affected complexly by changes in the speed of the bacteria or the fraction of motile bacteria, as well as their phototactic activity. Therefore, it is preferable to observe the behavior of individual
was teria in a light spot for determination of their phototactic response. The conclusion of Throm was confirmed by the present experiments in which antimycin, an inhibitor of photosynthetic electron transfer, prevented phototactic response. Motility of the bacteria was not affected by the inhibitor under aerobic conditions, indicating that the energy for motility is provided by respiratory metabolism, which is not affected by antimycin (data not shown), as well as by photosynthetic metabolism. The inhibitory effect of antimycin on both phototactic response and photosynthetic electron transfer was annulled by PMS (Table 1). The effect of PMS has been interpreted as the formation of a bypass around the blocking site of antimycin in the electron transfer pathway (29).

From these observations, it is inferred that the reversal of swimming direction after a sudden decrease in actinic light intensity is a consequence of the decrease in photosynthetic electron flow. Accompanying the decrease of electron flow is a change in the redox state of components of the electron transfer system and a decrease (depolarization) of membrane potential measured as an absorbance change of the carotenoid (Fig. 3). Both the reduction of cytochrome c2 and the decrease in membrane potential after the light was turned off were inhibited by antimycin. Thus, by considering only the action of antimycin on phototactic response, one cannot determine whether a redox change of electron transfer components or another event, such as depolarization of membrane potential, is important in the induction of a phototactic response in bacteria.

Valinomycin is an ionophore that increases the permeability of biological membranes to certain cations (K+, Rb+, and Cs+). In the presence of these cations, the electrochemical gradient across the membrane is affected. Intact cells of R. rubrum were insensitive to the antibiotic, presumably because of their intact outer membrane. However, when they were "properly" treated with EDTA, valinomycin plus 10 mM KCl inhibited both the phototactic response and the light-induced absorbance change of carotenoid (Table 2, Fig. 4) but not the redox changes of components in the photosynthetic electron transfer system (data not shown). The inhibitory effect of valinomycin on the bacterial phototactic response is probably due to depolarization of the membrane, not to permeability of any particular cation, since the phototactic response was affected by the ionophore only when the membrane was depolarized by the presence of both valinomycin and an interacting cation (Table 3).

The involvement of membrane potential in bacterial tactic response has been proposed by several authors (1). Recently, Szmelcman and Adler (31) reported that a chemotactic stimulus induces changes in membrane potential of E. coli cells. They discussed the possible role of ion fluxes in regulating flagellar rotation. The important role of ions in chemotactic responses was suggested by Ordal and Goldman (23-25). They reported that uncouplers of oxidative phosphorylation, inhibitors of electron transport, and permeable anions induce transient "tumbling"; that A23187, an ionophore for divalent cations, plus EDTA induces incessant "tumbling"; and that permeable cations inhibit "tumbling" in Bacillus subtilis. Ordal (23) proposed that binding of certain cations to the switch of a hypothetical chemotactic machinery inhibits "tumbling."

Two possible roles of the membrane potential in the phototactic response of R. rubrum are conceivable: (i) the tactic signal inducing reversal of swimming direction is the depolarization of membrane potential; (ii) a normal membrane potential is required for flagellar reversal to occur. Since we could not determine the role of membrane potential in the phototactic response, we present three alternative possibilities for the signal that induces the response: a change in the redox state of the components in the photosynthetic electron transfer system; uptake of H+ (decrease in ΔpH across the membrane); and depolarization of the membrane potential.

Bacterial motility was not paralyzed by valinomycin plus potassium (or rubidium). The relationship between membrane potential and cell motility will be discussed elsewhere (S. Harayama and T. Iino, manuscript in preparation).

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