Reduction-oxidation poise regulates the sign of phototaxis in *Chlamydomonas reinhardtii*

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Edited by Susan S. Golden, University of California at San Diego, La Jolla, CA, and approved May 26, 2011 (received for review January 12, 2011)

In many phototrophic microorganisms and plants, chloroplasts change their positions relative to the incident light to achieve optimal photosynthesis. In the case of motile green algae, cells change their swimming direction by switching between positive and negative phototaxis, i.e., swimming toward or away from the light source, depending on environmental and internal conditions. However, little is known about the molecular signals that determine the phototactic direction. Using the green alga *Chlamydomonas reinhardtii*, we found that cellular reduction-oxidation (redox) poise plays a key role: Cells always exhibited positive phototaxis after treatment with reactive oxygen species (ROS) and always displayed negative phototaxis after treatment with ROS quenchers. The redox-dependent switching of the sign of phototaxis may contribute in turn to the maintenance of cellular redox homeostasis.

Phototaxis is a behavior in which organisms move toward or away from directional light (“positive” or “negative” phototaxis, respectively). For motile phototrophic organisms such as green algae, switching between positive and negative phototaxis is crucial for maintaining the optimal light exposure for photosynthesis: They need to harvest light energy, but when light intensity is far above the saturation point of photosynthesis, they suffer from high-light stress (1). Thus, the direction or “sign” of phototaxis should be tightly controlled. The biflagellate unicellular green alga *Chlamydomonas reinhardtii* provides useful advantages for the study of the phototactic sign. Cells quickly change their swimming direction following light perception by channelrhodopsin (2). Many phototaxis-deficient mutants have been isolated, including a strain that exhibits only negative phototaxis [agg1 (phototactic aggregation), as discussed below].

Several extracellular factors have been shown to affect the sign of phototaxis in *Chlamydomonas* (3). Most importantly, light intensity controls the sign, which is positive under low light and negative under high light (4). Extracellular calcium concentration also affects the sign, an effect that is consistent with the calcium-dependent regulation of the beating balance between the two flagella (5, 6). However, even under light and medium conditions that are known to change the phototactic sign, there almost always is a population of cells that shows phototaxis of the opposite sign. In addition, after prolonged light stimulation, cells exhibit adaptation and become less sensitive to the light signal. Thus, the effect of the extracellular factors must vary depending on the internal condition of each cell. Factors that have been thought to affect the internal conditions include circadian rhythm and photosynthetic activity (7–9). Circadian rhythm dynamically affects the magnitude of phototaxis, but whether it also affects the sign of phototaxis is not known (7). In contrast, photosynthetic activity clearly affects the sign: Cells of a certain wild-type *Chlamydomonas* that show negative phototaxis under normal light conditions show positive phototaxis when treated with an inhibitor of photosynthesis, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) (8). The molecular mechanism underlying this phenomenon is unknown.

Eukaryotic cells possess multiple systems for maintaining the cytoplasm in a moderately reduced state called “reduction-oxidation (redox) homeostasis” (10). Because photosynthesis is

known to change the cytoplasmic redox poise greatly (11, 12), we surmised that redox balance might play a role in switching the sign of phototaxis. To test this hypothesis, we examined phototaxis in *Chlamydomonas* after artificially changing the cellular redox poise. We report here that the sign of phototaxis is, in fact, determined by the cellular redox poise.

Results

To change the *Chlamydomonas* cellular redox poise, we used two groups of membrane-permeable redox reagents: reactive oxygen species (ROS), which cause oxidative stress (group 1), and reagents that quench ROS and reduce oxidative stress (group 2). We used H₂O₂, tertiary butyl hydroperoxide (*t*-BOOH), and Neutral Red as group 1 and 4-hydroxy-2, 2, 6, 6-tetramethyl piperidine 1-oxyl (TEMPOL), dimethylthiourea (DMTU), and L-histidine as group 2. Cells were treated with these reagents at concentrations sufficient to cause oxidative stress (group 1) (13) or reductive stress (group 2) (14). Following reagent treatment, phototactic behavior was observed by positioning a green light-emitting diode (LED) ($\lambda = 525$ nm, ~ 50 μ mol photons m⁻² s⁻¹) adjacent to one side of the culture dish.

We observed both a wild-type and a negatively phototactic strain, agg1 (15). First, to observe the net movement of cells, cell suspensions were illuminated with directional green light. Wild-type cells showed positive phototaxis, whereas agg1 cells exhibited negative phototaxis (Fig. 1). However, after treatment with an ROS (H₂O₂), cells of both strains came to display positive phototaxis. Conversely, both strains came to show negative phototaxis after treatment with an ROS quencher (TEMPOL) (Fig. 1).

We next examined whether individual cells orient themselves to the direction of light, to ensure that the photo-accumulation was brought about by the cells' phototaxis rather than by a photophobic response. For this purpose, we measured the angle (θ) between individual cell's swimming direction and the light direction 15 s after the onset of stimulation with directional light (Fig. 2A). After treatment with two kinds of ROS, both wild-type and agg1 cells exhibited positive phototaxis; in contrast, after treatment with the two kinds of ROS quenchers, both strains exhibited negative phototaxis (Fig. 2B and Movies S1, S2, S3, and S4). The threshold concentrations of the ROS and ROS quenchers needed for the sign switching varied slightly from one experiment to another, presumably depending on the culture conditions. However, the difference in the threshold concentrations was always within $\sim 50\%$ of the concentrations indicated in Fig. 2B. Under all conditions, cells of oda1 (outer dynein arm), a mutant that lacks the redox-sensitive outer arm dynein (14, 16, 17), displayed almost the same flagellar beat frequency as the control cells (Table S1), indicating that the ROS or ROS

Author contributions: K.W. and R.K. designed research; K.W., Y.M., and S.M. performed research; K.W., Y.M., and S.M. analyzed data; and K.W. and R.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1100592108/-DCSupplemental.

Fig. 2. The effect of redox reagents on the phototactic sign was dose dependent and reversible. (A) Cell swimming direction was measured for 1.5 s following 15 s illumination with a green LED to avoid the effects of photophobic response occurring immediately after the onset of the LED illumination. (B and C) Polar histograms depicting the percentage of cells moving in a particular direction relative to light illuminated from the right, with or without treatment with redox reagents (12 bins of 30°; $n = 50$ cells per condition). Experiments were done at least three times per condition, and representative results are shown. (B) Both strains showed random swimming when not illuminated with a green LED. Wild-type cells exhibited positive phototaxis, and *agg1* cells exhibited negative phototaxis without treatment with any reagents. Note that wild-type cells exhibited negative phototaxis after treatment with ROS scavengers, whereas *agg1* cells showed positive phototaxis after treatment with ROS (histograms in yellow boxes). After washout of the reagents, each strain reverted to the same phototaxis direction as control cells, suggesting that the effects of the reagents are reversible. (C) Neutral Red (NR) and L-histidine (His), an inducer and a scavenger of singlet oxygen (a type of ROS), respectively, did not affect the sign of phototaxis.

manner. It is interesting that NAD- and NADP-dependent malate dehydrogenases are present in the flagellar membrane/matrix from the *Chlamydomonas* flagellar proteome database (26). Malate is a compound used indirectly to transport excess reducing equivalents from the chloroplast to the cytosol (27). Photosynthetic activity may change the redox poise of flagellar cytosol through the functions of those enzymes.

It also is possible, however, that the redox poise affects photoreception by modulating the eyespot or the subsequent signal transduction process that leads to a change in flagellar activity. As stated above, the phototactic behavior of a *Chlamydomonas* cell most likely is based on the turning of its swimming path, caused by a transient imbalance between the propulsive forces produced by the two flagella (28–30). Because the cell rotates around its long axis at a frequency of ~ 2 Hz while swimming, the two flagella alternate their positions periodically. Thus, the turning direction should reverse if the onset of the transient beating imbalance advances or delays by ~ 0.25 s. Such a delay or advance could be produced at any of the stages of signal transduction, which include photoreception, membrane depo-

larization, Ca^{2+} entry, and a change in the activity of the two flagella. A change in the photoreception step actually has been shown to cause a reversal of the steering direction (31). Thus, the photoreceptor may well be the target of the redox control. Detailed comparison of the movement and photoreception properties between positively phototactic cells (28) and negatively phototactic cells after treatment with redox reagents may provide some clues to the mechanism of the reversal of phototactic sign.

In conclusion, we have shown that the sign of phototaxis in *Chlamydomonas* is switched by the cytoplasmic redox poise that is changed by certain kinds of ROS. This redox-controlled switching of phototaxis direction may help maintain the redox homeostasis in the cytoplasm (but not in the chloroplast) via the regulation of photosynthesis. When the cytoplasm is oxidized, cells migrate toward light and increase photosynthesis activity, and thus the cytoplasm is reduced. When the cytoplasm becomes overly reduced, cells switch to negative phototaxis, the photosynthesis decreases, and the cytoplasm becomes oxidized. Such

Fig. 3. Phototactic indexes change significantly after treatment with certain types of redox reagents. The phototactic Index ($\pm$ SD) was calculated as an average value of $\cos\theta$ in Fig. 2 ($n = 50$). When cells are not illuminated and swim in random directions, the phototactic index should be ~ 0 . When 100% of cells show clear positive or negative phototaxis, the phototactic index is 1 or -1 , respectively. Asterisks indicate a significant differences from control (illuminated, no reagents) conditions ($P < 1.0 \times 10^{-16}$, t test). His, L-histidine; NR, Neutral Red.

regulation may function as a negative-feedback mechanism that maintains the moderately reduced state of the cytoplasm.

Materials and Methods

Cell Culture and Strains. *Chlamydomonas reinhardtii* strains CC124 (*nit1⁻* (nitrate reductase), *nit2⁻*, *agg1⁻*, *mt⁻* (mating type)) (15), CC125 (*nit1⁻*, *nit2⁻*, *mt⁻*) CC1168 (*y1⁻*, *mt⁻*) (20), and CC2228 (*oda1⁻*, *mt⁻*) (16) were used and are referred to as “agg1,” “wild type,” “y1a,” and “oda1,” respectively. Cells were grown in tris-acetate phosphate medium (32) with aeration at 20 °C on a 12-h/12-h light/dark cycle. The y1a cells were grown in 24-h darkness.

Reagents. DMTU, Neutral Red, Igepal CA-630, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. ATP, creatine kinase, and creatine phosphate were purchased from Roche Diagnostics. All other chemicals were purchased from Wako Pure Chemical Industries.

Phototaxis Assay. The phototactic behaviors of *Chlamydomonas* cells were quantified by recording and analyzing their behavior under continuous unidirectional illumination using a green LED ($\lambda = 25$ nm, ~ 50 μ mol photons $m^{-2} s^{-1}$). The photon flux density was measured with an LI-1000 Data Logger (LI-COR Inc.). The wavelength of the light source was selected to be close to the maximum absorption wavelength (500 nm) of *Chlamydomonas* channelrhodopsin 1, the photoreceptor functioning in phototaxis (33–35). Cells were washed with an experimental solution [5 mM Hepes (pH 7.4), 0.2 mM EGTA, 1 mM KCl, 0.3 mM $CaCl_2$] (22), shaken gently under white light for 1 h, and kept under dim red light for 15 min before the phototaxis assays.

Fig. 4. The redox reagents changed the phototactic sign of a chlorophyll-deficient mutant. The y1a strain has a defect in chlorophyll synthesis when grown in the dark. Dark-grown y1a cells showed strong negative phototaxis without treatment with redox reagents. Treatment of y1a cells with redox reagents changed the phototactic sign in the same manner as in agg1 cells. These data suggest that the phototactic sign was changed directly by the cytoplasmic redox poise, not by a change in photosynthetic activity.

For dish assays, cell suspensions ($\sim 10^7$ cells/mL) were put in petri dishes (30 mm in diameter, 10 mm thick), illuminated with a green LED from one side for 15 min, and photographed (DMC-LX2; Panasonic).

For single-cell analysis, petri dishes were placed on the stage of an inverted microscope (IX70; Olympus) and illuminated with a green LED. The behavior of the cells was observed with dim red light ($\lambda > 600$ nm, ~ 5 μ mol photons $m^{-2} s^{-1}$) and was video recorded using a CCD camera (XC-75; Sony). The angle (θ) between the light direction and the swimming direction was measured during 1.5 s following illumination with a green LED for 15 s. Images of swimming cells were auto-tracked using Image Hyper software (Science Eye). The angles were calculated from the cell trajectories.

Beat Frequency Analysis. Flagellar beat frequency was determined from the power spectra of cell body vibration signals in microscope images of a population of cells (36). Median frequency was obtained from the power spectra averaged for 20 s.

Reactivation of Demembranated Cell Models. Cell models were prepared and analyzed by the method of Kamiya and Witman (6), with modifications. Calcium buffers for reactivation were prepared according to Wakabayashi et al. (37), with modifications. In brief, after cells were washed with a buffer containing 10 mM Hepes (pH 7.4) and 4% sucrose, cells were demembranated with HMKP buffer [30 mM Hepes, 5 mM $MgSO_4$, 50 mM K-acetate, 1% polyethylene glycol (M_r 20,000)] containing 0.1% Igepal CA-630. Cell models were reactivated in four different reactivation buffers with either Ca^{2+} -free or Ca^{2+} buffer and with either reducing or oxidizing buffer. All buffers were made in the HMKP buffer containing 1 mM ATP, 70 U/mL creatine kinase, and 5 mM creatine phosphate. To change the concentration of free Ca^{2+} , either 1 mM EGTA or 5.2 mM EGTA plus 3.2 mM $CaCl_2$ [which results in a final free- Ca^{2+} concentration of $\sim 3.9 \times 10^{-8}$ M ($\sim pCa7.4$)] was added. These buffers are referred to as “ Ca^{2+} -free buffer” and “ $+Ca^{2+}$ buffer,” respectively. To change the redox state, either 1 mM DTT or 10 μ M DTNB (17) was added. These buffers are referred to as “reducing buffer” and “oxidizing buffer,” respectively. Cell models reactivated in either buffer solution were observed under a dark-field microscope. Cells that were rotating in small areas by predominantly beating one of the two flagella were chosen, and the dominance of the *cis*- or *trans*-flagellum (the flagellum closer to the eyespot or farther from the eyespot) over the other was determined by visual inspection (6).

ACKNOWLEDGMENTS. We thank Benjamin D. Engel for his critical reading of the manuscript, Takahiro Ide and Yuta Mitani for assistance with the phototaxis assays, and Drs. Ichiro Terashima and Ko Noguchi for the measurement of photosynthetic activity of *Chlamydomonas* cells and for helpful discussions. This work was supported by grants from the Japan Society for Promotion of Sciences and the Asahi Glass Foundation (to K.W.) and a by grant from the Ministry of Education, Culture, Sports, Science and Technology (to K.W. and R.K.).

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