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Fluorescence and Photosynthetic Activity of Chloroplasts in Acid and Alkaline Zones of *Chara corallina*

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Abstract—Continuous profiles of local pH near the cell surface of *Chara corallina* were recorded during uniform longitudinal movement of an internodal cell relative to a stationary pH microelectrode. Under illumination, the pH profile consisted of alternating acid and alkaline bands with a pH difference of up to 3 pH units. After darkening, the bands disappeared and pH became uniformly distributed along the cell length. Chlorophyll fluorescence of chloroplasts was measured by microfluorometry at different locations within one cell, and significant differences were observed in close relation to light-dependent pH banding. The chlorophyll fluorescence yield was lower in zones of low external pH than in alkaline zones both under actinic and saturating light. The fluorescence parameters F and F'_m and the quantum yield of photosystem II (PSII) displayed variations along the cell length in accordance with pH changes in unstirred layers of the medium. The results show that PSII photochemical efficiency and the rate of noncyclic electron transport are higher in the chloroplasts of acid zones (zones of H^+ extrusion from the cell) than in alkaline zones. The dependence of photosynthetic electron transport on local pH near the cell surface may result from different contents of CO_2 in acid and alkaline regions. The acid zones are enriched with CO_2 that readily permeates through the membrane providing the substrate for the Calvin cycle. Conversely, a poorly permeating form, HCO_3^- is predominant in alkaline zones, which may restrict the dark reactions and photosynthetic electron flow.

Key words: Chara corallina - acid and alkaline zones - chlorophyll fluorescence - proton fluxes - photosynthesis

INTRODUCTION

The internodes of characean algae, upon exposure to light, create alternating circular regions of proton extrusion and proton uptake [1–3]. The mechanism and the functional role of this process are scarcely known, despite the growing interest in the regulation of membrane transport and the self-organization phenomena in biological systems. The origin of acid and alkaline bands near the cell surface is closely related to the photosynthetic activity of chloroplasts and transport events at the plasmalemma [4, 5]. The transport properties of plasmalemma in darkened cells are uniform over the internode, but the onset of photosynthesis eliminates this homogeneity. The acid zones arise around plasmalemma regions where an ATP-dependent H^+ pump actively transports protons from the cytoplasm to the external medium [6, 7]. Conversely, no H^+ pump activity was observed in the alkaline zones. The alkaline zones seem to originate from the abrupt increase in H^+ conductance of the plasma membrane at high pH [8, 9], which favors the passive influx of protons into the cytoplasm and promotes the alkalization of the medium. Segregation of the membrane domains with active extrusion of protons and the domains with high H^+ conductance gives rise to extracellular local electric currents flowing from acid to alkaline regions [1].

Taking into account the light-dependent heterogeneity of plasmalemma in photosynthesizing cells, it seems reasonable to assume that chloroplasts adjacent to the plasmalemma domains with contrasting characteristics differ in functional activity. Such functional heterogeneity could, for example, provide the basis for the inhomogenous distribution of ATP within the cell and, thereby, account for higher rates of H^+ pumping in regions with higher ATP production. On the other hand, the pH shifts in unstirred layers affect the ratio of CO_2 and HCO_3^- content near the cell surface [10]. Unlike the anionic form (HCO_3^-), the neutral form (CO_2) readily permeates through the cell membranes. Therefore, the chloroplasts situated in acid and alkaline zones may experience different availabilities of the substrate used in the Calvin cycle.

Abbreviations: APW—artificial pond water; PAM—pulse amplitude modulation (fluorometer); PSII—photosystem II.

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The first attempt to investigate the relations between spatial heterogeneity of pH along *Chara corallina* cells and photosynthetic activity was undertaken by Plieth *et al.* [11]. These authors observed some differences between the induction curves of chlorophyll fluorescence in cell regions with proton-extruding and proton-absorbing activities. However, univocal interpretation of the results was complicated by drawbacks of the methods used for the detection of alkaline and acid bands. For example, the application of pH-indicating dye phenol red for the visualization of bands led to the nonuniform illumination of chloroplasts in different regions along the cell length (owing to light absorption by the alkaline form of the indicator). The alternative approach based on the use of cell-wall incrustations (calcium salt depositions) as indicators of the alkaline zones in old cells [11] is also inappropriate, because light scattering differs for calcified and noncalcified cell regions. With these methodical shortcomings, quantitative and qualitative comparison of fluorescence parameters recorded at different cell parts becomes a complicated matter.

Here we tried to verify the assumption that the spatial heterogeneity of functional activity is not only characteristic of the plasmalemma but is also specific for chloroplasts arranged in a one-layer network in close proximity to this membrane. We used pH microelectrodes for detecting acid and alkaline bands, since this method does not interfere with microfluorometric measurements. A methodically novel feature was the use of a microfluorometer with the pulse amplitude modulation system (Microscopy PAM) [12]. The PAM fluorometry permits the measurement of PSII photochemical efficiency and the rate of photosynthetic linear electron flow. Our findings demonstrate the spatial heterogeneity of fluorescence parameters of chloroplasts located within one illuminated internode. They also show that oscillations of photochemical efficiency along the cell length correspond to maxima and minima in the longitudinal pH profile.

MATERIALS AND METHODS

Experiments were performed on cells of characean alga *Chara corallina* Klein ex Willd. grown in an aquarium under laboratory conditions (scattered daylight, 20–22°C). Individual internodes measuring about 6 cm in length and ~1 mm in diameter were excised and kept in artificial pond water (APW) containing 0.1 mM KCl, 1.0 mM NaCl, and 0.1 mM $CaCl_2$. Prior to the experiment, the cell was placed in an experimental chamber filled with APW. The chamber was mounted on either the movable table controlled by a C-804 DC-Motor Controller (Physik Instrumente GmbH, Germany) or the stage of an Axiovert 25 inverted microscope (Zeiss, Germany). To ensure simultaneous measurements of fluorescence and pH in the regime of scanning along the cell, the chamber with an internode was mounted on a movable table (Biopribor, Pushchino, Russia) providing uniform movement with a velocity of approx. 130 μm/s at discrete steps of 0.1 μm. We used the cells without apparent signs of calcification. Experiments were conducted at room temperature.

Alkaline and acid bands were identified with glass-insulated antimony pH-microelectrodes having a tip diameter of about 50 μm [13]. The potential of pH microelectrode as a function of pH was calibrated relative to an Ag/AgCl reference electrode with the use of standard buffer solutions (Orion, United States). The slope of the electrode characteristics was about 58 mV/pH unit. The potential difference between the pH microelectrode and the reference electrode was measured with a VAJ-51 electrometric amplifier (Germany). The tip of the pH probe was positioned with a micromanipulator at a distance of 50–100 μm from the cell surface. This distance was permanently controlled by fine readjustment during uniform movement of the cell relative to the stationary pH probe.

Chlorophyll fluorescence from small portions of an intact cell was measured with a Microscopy-PAM fluorometer (Walz, Germany). Characteristics and features of this recently developed device were described by Goh *et al.* [12]. The basics of PAM fluorometry and the saturation pulse method were presented in several publications [14, 15]. The measuring light and saturation light pulses were provided with a NSBG 500 light-emitting diode (Nichia, Japan) emitting in the blue spectral range. The separation of chlorophyll fluorescence from excitation light was accomplished by the filter combination supplied with Microscopy PAM as described in [12]. Excitation light passed through a BG 39 blue filter (Schott, Germany) and was directed to the object by a light-splitting plate (R65, Balzers, Liechtenstein). Chlorophyll fluorescence was directed to a photomultiplier via a dichroic filter (DT Yellow, Balzers) and a red filter (RG 645, Schott, Germany). This filter combination cut off the scattered blue light. Signals of fluorescence and pH were digitized with a 1401 Plus analog–digital converter (CED, United Kingdom) and recorded on a computer.

For steady-state fluorescence measurements, we used dark-adapted cells and cells illuminated with blue actinic light. The potential photochemical yield of PSII in dark-adapted cells was determined by the following formula: $Y = (F_m - F_o)/F_m$, where F_o is the minimal fluorescence yield under weak measuring light and F_m is the maximum fluorescence yield observed under the action of a saturation pulse [15]. The PSII effective yield in cells exposed to actinic light was estimated from the equation $Y' = (F'_m - F)/F'_m$, where F'_m is the maximum fluorescence obtained under a saturation pulse and F is the actual fluorescence yield measured under actinic light [14, 15]. The F'_m level is usually less than F_m owing to the development of nonphotochemical quenching.

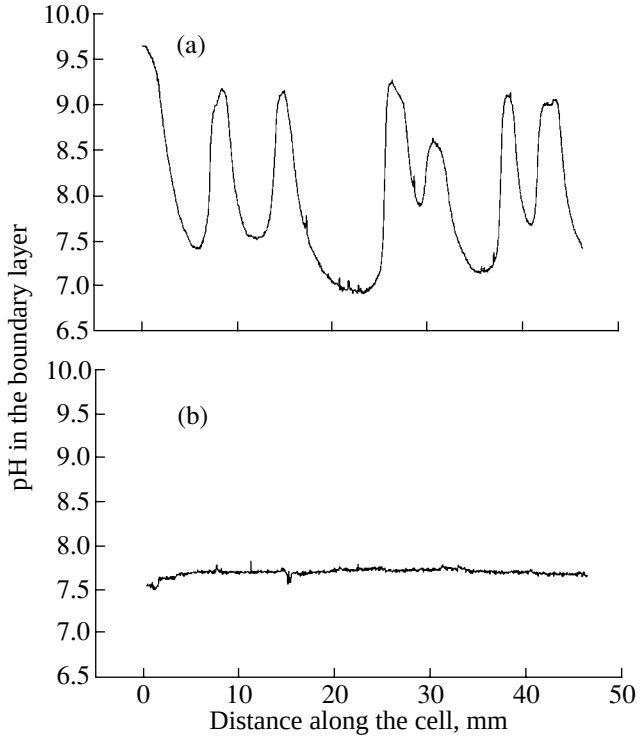

Fig. 1. Longitudinal profiles of pH near the surface of *C. corallina* (a) under illumination (100 W/m^2) and (b) 40 min after transferring the cell to darkness. The pH values were measured at a distance of 50 μm from the surface during gradual movement of the cell relative to pH electrode at a velocity of 200 μm/s.

Actinic light was provided by a halogen lamp from a built-in light source of an Axiovert 25 inverted microscope. This light source was positioned over the movable table with the *Chara* cell. In some experiments, a slide projector was used as a light source. Actinic light passed through a SZS-22 blue glass filter ($\lambda < 580$ nm); the light intensity at the cell level was about 5 W/m^2. Blue light of such intensity did not interfere with microfluorometric measurements, because yield determination in PAM-type fluorometers is based on fluorescence responses to modulated light. However, a considerable increase in actinic light intensity above this level reduced the signal-to-noise ratio (the noise increases with a direct current component flowing through a photomultiplier).

Simultaneous measurements of pH and actual fluorescence F were performed while gradually moving the cell at a velocity of ~130 μm/s. Simultaneous measurements of pH and fluorescence parameters F and F_m' were performed at discrete locations along the cell length. At each location, two saturation pulses were applied with a separation time of about 20 s, and F_m' was determined. Fluorescence was detected from a circular area with a diameter of about 100–200 μm. The objective lens was focused on the nearest layer of chloroplasts in the bottom half of a cell, since the fluorescence signal of this layer was substantially higher than that of the distant chloroplast layer in the upper half of the cell.

To detect possible differences in photosynthetic oxygen evolution between alkaline and acid zones we used a glass-insulated Pt microelectrode. The diameter of the Pt wire was 0.1 mm. The potential of the Pt cathode was set at 0.65 V with respect to the Ag/AgCl reference electrode. The current was measured with a Nano-amperemeter (Knick, Germany) and digitized with an analog—digital converter. The current–voltage relationship showed a typical plateau near 0.65 V, with the plateau current sensitive to oxygen concentration. The depletion of oxygen by adding crystals of sodium dithionite eliminated this current. The plateau current was independent on the pH in the range 7.0–10.0.

Experiments were performed in at least four replicates on different cells. The data shown in the figures contain the results of representative experiments.

RESULTS

Figure 1 shows longitudinal profiles of the external pH in the vicinity of a *C. corallina* cell measured in bright light (irradiance 100 W/m^2) and in darkness. The profiles were obtained by a continuous displacement of the cell relative to the pH microelectrode at a velocity of 200 μm/s. Lucas and Nuccitelli [1] applied pH microelectrodes for the detection of acid and alkaline zones, but their records contained few measurement points and did not provide sufficient spatial resolution. Our technique is the first example of continuous measurements of longitudinal pH profiles. The transfer of a cell to darkness for 30 min or longer resulted in the almost full disappearance of the bands in the pH profile (leveling of pH over the internode). Subsequent prolonged illumination of the cell (for 1 h) led to the restoration of the nonuniform regular pH profile with alternating acid and alkaline bands.

Under actinic illumination of a *Chara* cell, chlorophyll fluorescence parameters substantially differed for proton-extruding and proton uptake zones (acid and alkaline zones, respectively). The actual fluorescence level F was lower in acid zones than in alkaline regions. The difference was as high as 50–80% with respect to the fluorescence yield in acid zones. The values of maximum fluorescence F_m' attained during saturation light pulses were also lower in the acid regions and comparatively high in the alkaline zones. The difference (of about 30%) significantly exceeded standard deviations in serial assays of fluorescence at the same location (1.8 and 1.0% for F and F_m', respectively).

Figure 2 shows the results of simultaneous measurements of local pH near the cell surface and fluorescence F under actinic illumination. The profiles were measured while continuously moving the cell relative to the tip of the pH probe and the fluorescence-collecting lens. It is seen that the profile of chlorophyll fluores-

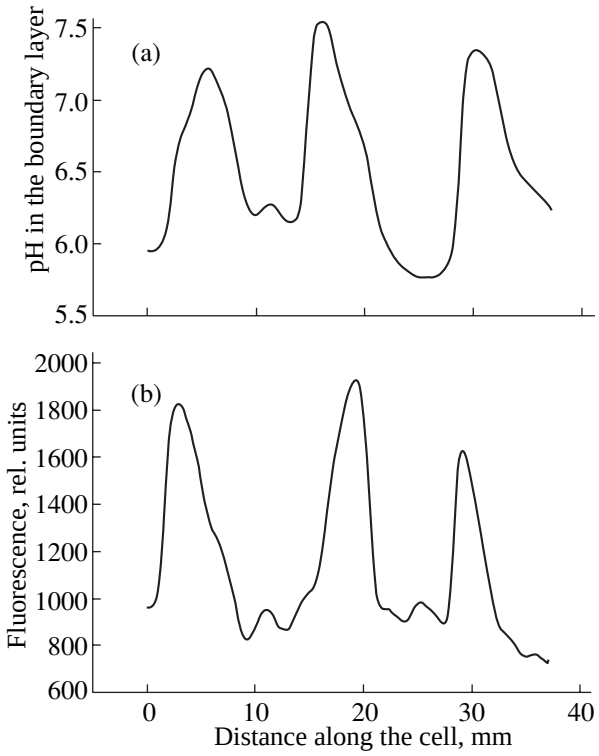

Fig. 2. Simultaneous measurement of longitudinal profiles of (a) pH and (b) fluorescence yield F in *C. corallina* cell exposed to overall illumination with blue light (5 W/m^2). During measurements, the cell was gradually moved relative to the objective lens of the microfluorometer and the pH microelectrode at a velocity of 130 μm/s.

cence along the cell length is nonmonotonous and contains alternating peaks and minima. Furthermore, the position of peaks roughly coincides with the position of alkaline bands in the pH profile.

Using values of F and F'_m for different cell parts, the photochemical efficiency of PSII (Y') was calculated [14, 15] and plotted as a function of the distance along the cell. Figure 3 shows the results of simultaneous measurements of parameter Y' (photochemical efficiency of PSII) and the local pH in unstirred layers near the cell surface. As seen from the curves presented in Fig. 3a, the highest values of PSII effective yield (Y' of about 0.45) were noticed in regions with low local pH, whereas the lowest values of Y' (0.25–0.27) corresponded to alkaline zones (pH 8.8–8.9).

Our data suggest a correlation between the photochemical efficiency of PSII and local pH values near the cell surface. Using the paired values of pH and Y', measured at different parts of the cell, we drew the plot relating these values (Fig. 4). As seen from this figure, the PSII photochemical efficiency decreases with the increase in local pH from 7.0 to 9.0.

Figure 3b shows the results of a similar experiment (see Fig. 3a) performed on the same cell 30 min after darkening. Under these conditions, the pH profile was smoothed away, similarly to what is shown in Fig. 1. The oscillations of PSII photochemical efficiency (Y') along the coordinate also disappeared. Thus, the cessation of photosynthesis resulted in the dissipation of the nonuniform spatial distribution of pH, fluorescence,

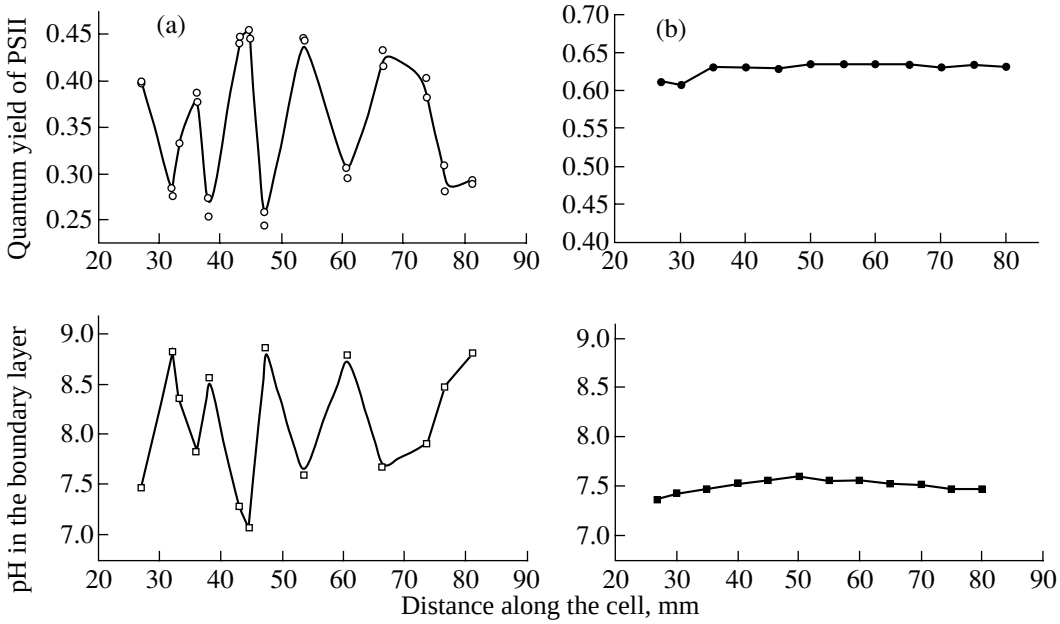

Fig. 3. Simultaneous measurements of the effective yield of PSII and pH near the cell surface of *C. corralina* (a) under illumination with blue light (5 W/m^2) and (b) 30 min after transferring the cell to darkness. The quantum yield of PSII was calculated from the formula $Y' = (F'_m - F)/F'_m$.

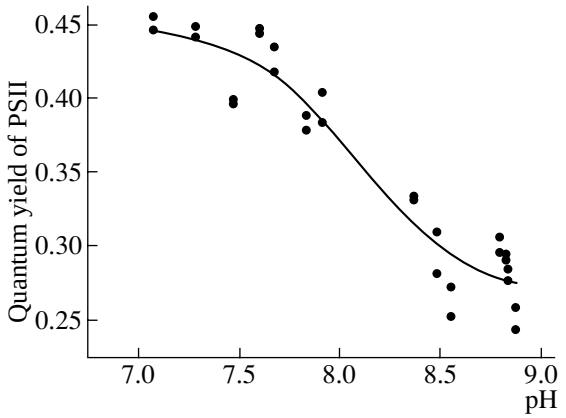

Fig. 4. Dependence of PSII quantum yield on pH in the boundary layers of the medium. Symbols signify experimental values measured in the acid and alkaline zones of *C. corallina* cells; the sigmoid curve shows the approximation of experimental data.

and PSII photochemical yield (*Y*') to a homogeneous state.

These results show that differential photochemical and fluorescence performance of chloroplasts in cell zones with proton-pump and proton-sink activities (acid and alkaline zones, respectively) is not a permanent feature related to morphological distinctions (e.g., different number of chloroplasts per unit area). These distinctions along the cell fade upon the disappearance of pH banding.

In order to reveal possible differences in photosynthetic activity between chloroplasts located in acid and those located in alkaline zones, we performed amperemetric measurements in these zones using an open Pt microelectrode. In these experiments, we first checked the occurrence of pH banding. Next, we measured the longitudinal profile of the current, which was presumably related to oxygen reduction on the Pt cathode. As shown in Fig. 5, the profiles of the current and pH measured on the same cell were strongly correlated. The peaks of the current (oxygen concentration) were observed in the regions of low pH, whereas the lowest current values were attributed to alkaline zones. The difference between maxima and minima of current reached as much as 30% with respect to the current level in the alkaline zones. In the control experiment, when the potential of the Pt cathode was set to 0 mV, no current flowed in the measuring circuit, and the axial displacement of the cell relative to the Pt electrode was not accompanied by any current oscillations.

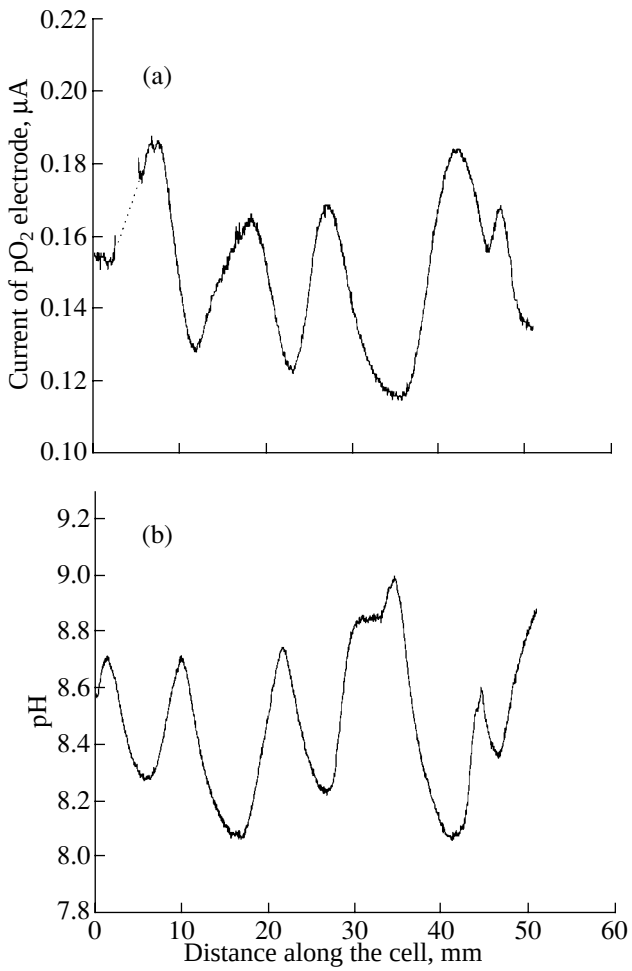

Fig. 5. Comparison of longitudinal profiles of (a) pO_2 (current of open Pt microelectrode) and (b) pH in the boundary layers of medium on *C. corallina* cell. The pH and pO_2 profiles were measured at the velocity of cell movement of 200 μm/s. Light intensity was 100 W/m^2 (full light of incandescent lamp).

DISCUSSION

The origin of regularly alternating acid and alkaline bands in the unstirred layers of the medium around the cells of characean algae was the focus of several publications [1, 2, 5]. The main method used in these studies was based on measurements of extracellular local currents with the vibrating electrode [1, 3, 5]. The local circular currents flowing from the site of H^+ extrusion (acid zones) to the sites of H^+ influx into the cytoplasm (alkaline zones) create a voltage drop at the water layers near the cell, which can be measured with a vibrating electrode. The drawback of this method is the stirring of the solution by electrode vibration, which disturbs diffusion gradients near the cell surface. Local circular currents were also discovered in growing roots [16]. Despite a certain similarity between extracellular current patterns arising in algal cells, roots, and other objects, it should be noted that the formation of functionally different ion-transporting domains in the plasmalemma of *Nitella* and *C. corallina* cells is specific for self-organization phenomena related to photosynthesis. This relationship includes at least two aspects. On the one hand, photosynthetic phosphorylation produces ATP required for the operation of the electro-

genic H^+ pump of plasmalemma and for the maintenance of circular local currents (H^+ flow) between acid and alkaline zones. On the other hand, the nonuniform distribution of pH along the axis of a cylindrical cell may have an effect on the photosynthetic activity of chloroplasts in the respective zones. This could be mediated, for example, by different contents of CO_2 in acid and alkaline zones. The acid–base equilibrium between CO_2, HCO_3^-, and CO_3^{2-} includes two stages with $pK_1 = 6.35$ and $pK_2 = 10.33$. The neutral form (CO_2), freely permeating through the membranes, is only available in acid zones, whereas the alkaline zones are enriched with poorly permeating charged forms [10]. Therefore, under CO_2 deficiency, the rate of photosynthetic electron flow can be restricted by the limited activity of Calvin cycle.

Our method of detecting the alkaline and acid regions in combination with measurements of chlorophyll fluorescence yield at small parts of a chloroplast layer in an individual cell allowed us to reveal significant distinctions between acid and alkaline zones in terms of the fluorescence parameters and the photochemical activity of PSII. A comparatively high fluorescence yield, which is characteristic of alkaline zones (Fig. 2), indicates that photochemical efficiency could be lower in these zones, since photochemical trapping is competitive to fluorescence and nonphotochemical quenching. Remarkably, the placement of a cell in darkness resulted in the concurrent leveling of pH and fluorescence profiles. Although the number and position of bands in the pH and fluorescence profiles measured under gradual axial movement of cell were similar in a rough approximation (Fig. 2), the shapes of these bands were different. This discrepancy can arise, for example, because the quantity *F*, unlike the dimensionless parameter *Y*', is sensitive to the number of chloroplasts in the area of fluorescence detection. Therefore, slight fluctuations in *F* may reflect the inhomogeneity of chloroplast distribution over the cell. The difference in the shapes of pH and fluorescence profiles can also be due to a nonlinear relation between fluorescence and pH upon a large-scale change in external pH, as well as to slight spatial divergence between the sites of pH and fluorescence detection. Furthermore, the actual structure of acid and alkaline zones can deviate from the ideal ring-shaped form.

It should be emphasized that spatial oscillations of photochemical efficiency cannot be due to inhomogeneous distribution of chloroplasts in different cell parts, because these oscillations were only evident on illuminated cells and disappeared in darkness (Fig. 3). Moreover, the photochemical efficiency *Y*' (a dimensionless parameter) is insensitive to the density of the chloroplast array. The light–dark transitions have no immediate effect on the density of chloroplast distribution in *C. corallina* cells since chloroplasts, in forming a single-layer array in the ectoplasm, remain immobile despite the intense streaming of the inner cytoplasmic layer (cyclosis) [17].

Our data support the proposal of Plieth *et al.* [11] that the chloroplasts situated in acid regions experience a sufficient supply of CO_2 as a substrate for the Calvin cycle, whereas chloroplasts of alkaline regions may experience a deficiency of CO_2. The shortage of substrate should inhibit the activity of PSI. This would result in the reduction of acceptor Q_A and the respective increase in the fluorescence yield, as well as in the suppression of the photochemical efficiency of PSII and the deceleration of noncyclic electron transport. Data presented in Fig. 3 directly support the assumption that the photochemical efficiency of PSII is higher in acid zones than in alkaline zones of a cell. Since the electron flow through PSII and PSI should be equal under a steady state, the parameter *Y*' can serve as an indicator of the linear electron transport rate in photosynthesis [14]. Thus, our results provide evidence that the photosynthetic electron transport rate is higher in the acid zones than in alkaline regions. The results of amperemetric measurements (Fig. 5) are consistent with the notion that photosynthetic activity (O_2 evolution rate) is higher in the acid zones than in alkaline zones.

In the boundary layers of the external medium, the fraction of CO_2 in the overall content of $HCO_3^- + CO_2$ at $pH < 9$ should be described by the expression $(1 + 10^{pH - pK})^{-1}$, where $pK = 6.35$. The observed dependence of PSII photochemical efficiency in relative units $(Y' - Y'_{min})/(Y'_{max} - Y'_{min})$ versus the pH of the external medium is described by a similar formula with different coefficients: $(1 + 10^{a(pH - pK)})^{-1}$, where $pK = 8.1$ and $a = 1.67$. The above difference can result from a number of causes, such as the involvement in carbon supply of intracellular compartments having pH different from pH of the medium (cytoplasm, chloroplast stroma) and nonlinear dependence of the CO_2 fixation rate on the CO_2 concentration. The cell wall may exert a certain influence owing to its ion-exchange and buffer properties. Conversely, the alteration of acid and alkaline zones along the cell length may induce local heterogeneity in the mechanical properties of cell walls (elasticity and extensibility). One of the intriguing questions is whether or not the spatial separation of proton-pump and proton-sink activities in *Chara* cells is reflected by the nonuniform distribution of pH within the cytoplasm along the cell length. Attempts to discover possible spatial patterns of cytoplasmic pH, the functional heterogeneity of chloroplasts and other organelles, and the regulatory mechanisms controlling transport systems in photosynthesizing cells represent a promising line for further investigation.

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