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Detection and characterization of acidic compartments (vacuoles) in *Chlorella vulgaris* 11h cells by ^{31}P-in vivo NMR spectroscopy and cytochemical techniques

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Abstract. Acidic inorganic phosphate (Pi) pool (pH around 6) was detected besides the cytoplasmic pool in intact cells of *Chlorella vulgaris* 11h by ^{31}P-in vivo nuclear magnetic resonance (NMR) spectroscopy. It was characterized as acidic compartments (vacuoles) in combination with the cytochemical technique; staining the cells with neutral red and chloroquine which are known as basic reagents specifically accumulated in acidic compartments. Under various conditions, the results obtained with the cytochemical methods were well correlated with those obtained from in vivo NMR spectra; the vacuoles were well developed in the cells at the stationary growth phase where the acidic Pi signal was detected. In contrast, cells at the logarithmic phase in which no acidic Pi signal was detected contained only smaller vesicles that accumulated these basic reagents. No acidic compartment was detected by both cytochemical technique and ^{31}P-NMR spectroscopy when the cells were treated with NH_4OH. The vacuolar pH was lowered by the anaerobic treatment of the cells in the presence of glucose, while it was not affected by the external pH during the preincubation ranging from 3 to 10. Possible vacuolar functions in unicellular algae especially with respect to intracellular pH regulation are discussed.

Key words: Acidic compartments – *Chlorella vulgaris* – Chloroquine – Compartmentation – Inorganic phosphate – Intracellular pH – In vivo nuclear magnetic resonance – Neutral red – Unicellular algae – Vacuoles

A lot of work has recently been done on the central vacuoles of higher plants and yeast cells, and their functions such as accumulation and degradation of macromolecules, exchange of ions and metabolites with cytoplasmic constituents have been understood. Thus, the vacuoles are assumed to take an important part in keeping the homeostasis of cytoplasm with respect to intracellular pH, concentration of metabolites, etc. In plant cells, ^{31}P-NMR spectroscopy has contributed to the estimation of cytoplasmic and vacuolar pH (Roberts 1984). The central vacuole of internodal cells of Charophyta has also been well studied physiologically as a model system of vacuoles (Moriyasu et al. 1984). The vacuoles of these systems play a static role as large spacers with an inert and metabolically cheap building material in addition to the dynamic functions (Boller and Wiemken 1986). From this point of view, we may assume that the vacuoles of these systems are specifically evolved to be large. In order to understand the feature, fundamental functions, and the evolutional aspects of the organelle, it is important to characterize the vacuoles in simpler plant systems.

Unicellular algae, such as *Chlorella* and *Chlamydomonas*, are ideal model systems for plant cell biology, since they have simple systems (1 cell-1 chloroplast), and extracellular growth conditions can easily be controlled. They can be cultured autotrophically and heterotrophically. They are also good systems for the in vivo NMR technique, because they can be treated as homogeneous cell suspension. Biochemical, genetical, and cytological studies can also be effectively carried out. Thus, unicellular algae are especially suitable systems for the study of vacuoles.

Atkinson et al. (1974) reconstructed the three-dimensional intracellular stucture of *Chlorella fusca*. According to them, the vacuoles containing polyphosphate granules occupied 8–13% of the total cellular volume, whereas over 90% volume was occupied by the central vacuoles in higher plants and Charophyta. They also showed some morphological changes during the cell cycle.

However, there is only a few works on the intracellular compartmentation and physiology of vacuoles in unicellular algae. Sianoudis et al. (1984, 1985) detected a small Pi signal at a field higher than the main cytoplasmic Pi signal in *Chlorella fusca* and assigned the former signal as the vacuolar Pi because of its lower pH value (pH 5.6). On the other hand, Mitsumori and Ito (1984) did not detect the vacuoles in ^{31}P-NMR spectra of *Chlorella vulgaris* 11h cells and assumed that vacuoles were negligible in that species. Gehl and Colman (1985) also reported that *Chlorella saccharophila* cells were nonvacuolate cells. Therefore, the presence of vacuoles in unicellular algae is not conclusive. For this reason, intracellular structural analysis in combination with in vivo NMR measurements is needed.

In the present work, cytochemical methods as well as in vivo NMR technique were applied to living *Chlorella vulgaris* 11h cells. Structure, development and possible functions such as pH regulation of this organelle under

Non-standard abbreviations. EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MDP, methylene diphosphonic acid; NMR, nuclear magnetic resonance; PCA, perchloric acid; PCV, packed cell volume; Pi, inorganic phosphate; Pic, cytoplasmic inorganic phosphate; Piv, vacuolar inorganic phosphate; ppm, parts per million; SP, sugar phosphates; TCA, trichloroacetic acid

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various growth stages and cellular conditions were discussed.

Materials and methods

Algal culture

Chlorella vulgaris 11h cells (Algal Culture Collection, University of Göttingen, FRG) were grown photoautotrophically with constant bubbling of air containing 1.5% CO_2 in flat oblong vessels under continuous illumination (10 klux) at 30° C. The culture medium contained 5.0 g KNO_3, 2.5 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.1 g K_2HPO_4, 2.8 mg $FeSO_4 \cdot 7H_2O$, and 1 ml of Arnon's "A_5" solution per liter (pH 5.7) (Ogasawara and Miyachi 1970) except that the content of Mn^{2+} was reduced to one tenth to avoid broadening of NMR signals (Mitsumori and Ito 1984). Unless otherwise stated, the cells were harvested after the growth for 1 week, when the cell density attained about 7 µl PCV/ml suspension.

Preparation of cells for ^{31}P-NMR measurements

Just before the NMR measurements, cells were washed with 2 mM K_2SO_4 solution twice and resuspended in 20 mM HEPES-KOH buffer (pH 7.6) at a cell density of 0.4 ml PCV/ml (total volume, 5 ml).

In order to investigate the effects of NH_4OH and medium pH on the pH of intracellular compartments, cells washed with K_2SO_4 were suspended in a solution described in the figure legend of Figs. 1 and 3 at a cell density of 4% PCV, and aerated by shaking at 30° C. For the anaerobic treatment, cells which had been shaken for 15 h in 2 mM K_2SO_4 were suspended in N-free culture media with or without enrichment of 0.2% glucose (cell density, 1% PCV), and bubbled with N_2 gas for another 150 min at 30° C.

The pH-titration of Pi chemical shift was performed by the method of Ohmori et al. (1986) with some modifications. Cells were extracted with 6 M perchloric acid and with distilled water (final concentration of PCA, 1 M). The cell extracts were frozen at − 20° C overnight, thawed, roughly neutralized with potassium bicarbonate, incubated on ice for 30 min, and centrifuged at 2,300 × *g* for 10 min. The pellet containing potassium perchlorate was discarded. EDTA·2Na (final, 2 mM) was added to the supernatants and the pH values were adjusted with NaOH and HCl. After the NMR measurement, the differences in the chemical shift values between the Pi and the external standard, methylene diphosphonic acid (MDP) at various pH values were plotted.

For preparation of TCA-treated cell suspension, cells were treated with TCA (final concentration, 8%), and kept on ice for 1 h. The cell suspension was neutralized with NaOH, followed by the addition of EDTA·2Na (final, 2 mM) (Oh-hama et al. 1986).

^{31}P-NMR measurements

^{31}P-NMR spectra were measured on a JEOL GX 400 spectrometer operating at 162.2 MHz in the pulsed Fourier transform mode without proton decoupling and unlocked at 27° C. The flip angle was 45°. The pulse repetition time was 0.52 s, and 500 scans were accumulated for each spectrum with the capped sample tube (diameter: 15 mm) spinning. As an external standard, 1.8% (w/v) MDP solution was sealed in a glass capillary tube and measured simultaneously. Chemical shifts were expressed in ppm relative to 85% phosphoric acid.

Cytochemical staining of vacuoles

(1) Neutral red. Cells were incubated in 50 mM HEPES-KOH buffer (pH 7.6) containing 10 mM neutral red (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for 10 − 60 min, and observed with a light microscope (Olympus).

(2) Chloroquine. Cells were incubated in 50 mM HEPES-KOH buffer (pH 7.6) containing 10 mM chloroquine (Sigma, St. Louis, MO, USA) for 10 − 60 min, washed with 2 mM K_2SO_4, and observed with Olympus fluorescence microscope with an interference filter transmitting 400 − 610 nm light (Heraeus Vakuumtechnik GmbH, Hanau, FRG).

Results

Detection and pH measurement of acidic compartments in intact cells by ^{31}P-NMR

Figure 1a shows Pi and sugar phosphate region of ^{31}P-NMR spectrum in intact *Chlorella* cells. There are two signals in the Pi region. Neither of them was affected by the addition of $MnCl_2$ (final concentration, 100 µM; data not shown), while the signal of extracellular Pi added disappeared due to the broadening effect by the paramagnetic ions (data not shown, Labotka and Kleps 1983). These results indicate that the two signals arose from phosphates localized within the cells.

In contrast to the in vivo spectrum of *Chlorella* cells (Fig. 1a), only one peak appeared in the orthophosphate region in TCA treated cell suspension (Fig. 1b) and PCA extract (data not shown). This supports the assumption that both peaks in the in vivo spectrum (Fig. 1a) arose from Pi in different intracellular compartments. From the pH titration curve on the chemical shift of the Pi signal in the PCA extract, the pK value was estimated as 6.9, and the total difference in the chemical shift was about 2.5 ppm. These values are in good agreement with reported values for Pi (Mimura and Kirino 1984; Sianoudis et al. 1984). From these results, we concluded that the two signals in the intact cells represent Pi's at pH values of 7.1 ± 0.2 and 6.3 ± 0.2. The neutral main signal represents the cytoplasm.

When the cells were incubated with 5 mM NH_4OH, the higher field Pi signal disappeared, while the lower-field Pi signal became higher (Fig. 1c). This indicates that the ammonium treatment made the pH-difference between the two compartments too small to be distinguished by this method.

Cytochemical stain of vacuoles

The intracellular distribution of acidic compartments were studied with neutral red and chloroquine, which are known as basic reagents specifically accumulated in acidic compartments (lysosomes, vacuoles, etc.) (Poole and Ohkuma 1981; Lenz and Holzer 1984). When neutral red was applied to cells at the stationary growth phase, several red-colored small spherical vesicles were observed in each cell (diameter: about 0.1 − 1 µm) (dark spots in Fig. 2a). They had nearly

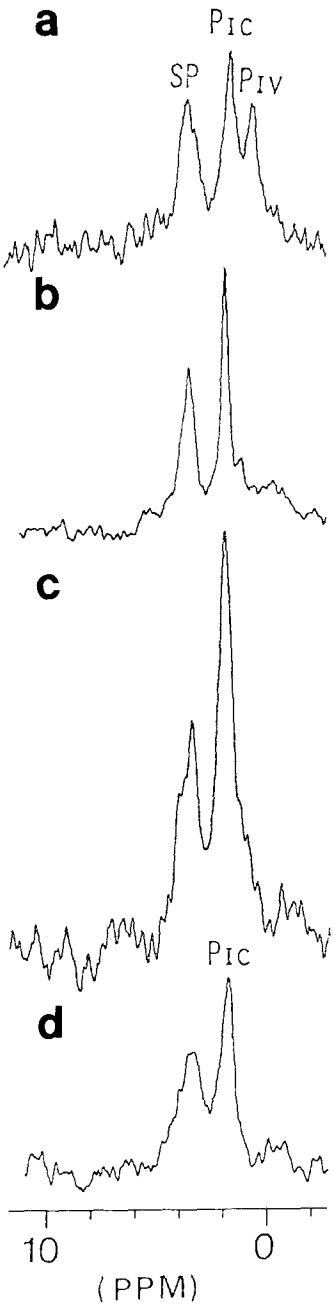

Fig. 1a–d. ^{31}P-NMR spectrum of intact *Chlorella* cells at the stationary growth phase (**a**), those cells treated with TCA (**b**), those cells after incubation with NH_4OH (the cells were shaken in a medium containing 2 mM K_2SO_4 and 5 mM NH_4OH for 15 min) (**c**), and intact cells at the logarithmic growth phase (**d**)

the same shape and volume as the vacuoles observed by electron microscope in *Chlorella fusca* cells (Atkinson et al. 1974). The pKa value of neutral red is 6.7 and the color is red only when the pH is below 6.8 (Conn 1969). Therefore, we may estimate that the pH values of these organelles are below 6.8. The same cells stained with chloroquine had the vesicles almost the same in size and shape, which were clearly observed as spots with light-blue fluorescence (white spots in Fig. 2b). These results indicate that acidic vesicles in the cells of *Chlorella vulgaris* 11h at the stationary growth phase are the vacuoles. We also observed fast movement and rotation of the vacuoles in the living cells.

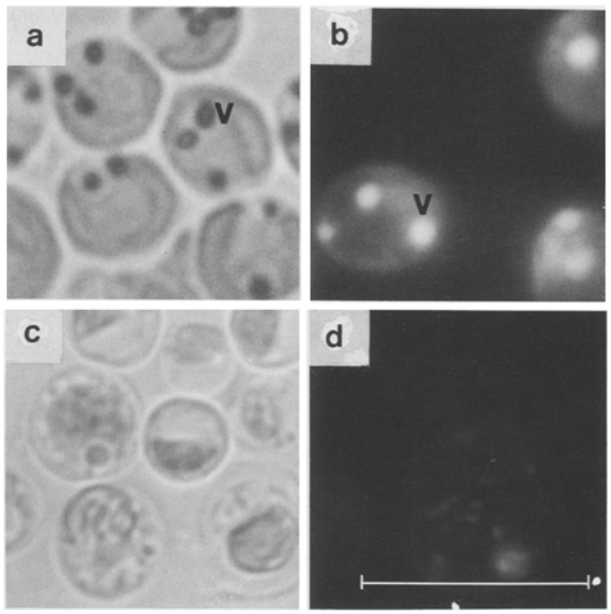

Fig. 2a–d. Cytochemical staining of vacuoles in living *Chlorella* cells (bar = 10 μm). **a** Cells at the stationary phase stained with 10 mM neutral red. **b** Fluorescence micrograph of the cells at the stationary phase stained with 10 mM chloroquine. **c** Cells at the stationary phase stained with 10 mM neutral red after the incubation with 10 mM NH_4OH. **d** Fluorescence micrograph of the cells at the logarithmic growth phase stained with 10 mM chloroquine. The photographs of *Chlorella* cells treated with neutral red (**a**, **c**) were taken with ca. 520 nm-light through an interference filter (Olympus G520), where red-colored vacuoles accumulating neutral red appeared as black dots (V, in the figure). This is due to the fact that the molecular absorbance of neutral red at this wavelength is six times more intense at acidic pH than at weakly basic pH. Fluorescence micrographs (**b**, **d**) were taken with an interference filter, which cuts off red light to eliminate the red fluorescence of chlorophyll

Table 1. Effects of various treatments on cytoplasmic and vacuolar pH values in intact cells of *Chlorella vulgaris* 11h, which were deduced from in vivo ^{31}P-NMR spectra

	Cytoplasmic pH[d]	Vacuolar pH[d]
Logarithmic phase cells		
1) Control[a]	7.2	n. d.
2) N_2-bubbled[b]	7.1	n. d.
Stationary phase cells		
1) Control[a]	7.1	6.3
2) N_2-bubbled[b]	7.1	6.4
3) NH_4OH-treated[c]	7.1	n. d.
4) N_2-bubbled[b] with glucose	6.8	5.7
5) 4) + NH_4OH[c]	7.1	n. d.

n. d. not detectable
[a] Cells were shaken in 2 mM K_2SO_4
[b] Time of anaerobic treatment, 150 min
[c] Cells were shaken in a medium containing 2 mM K_2SO_4 and 5 mM NH_4OH for 15 min
[d] Standard errors, about ± 0.2 in pH units

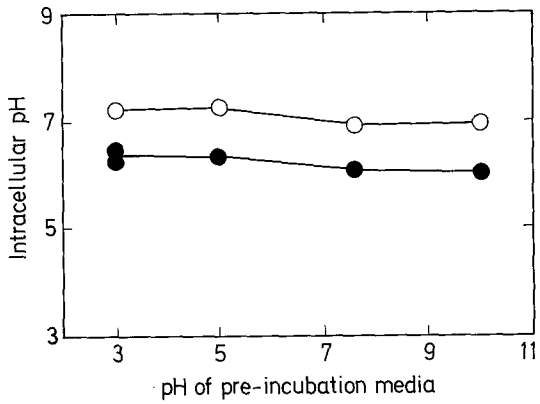

Fig. 3. Effects of pH of suspending media on intracellular pH in intact *Chlorella* cells. Cells were incubated for 2 h in a buffer solution (citrate buffer for pH 3–5, HEPES-KOH for pH 7–8, Tris-HCl for pH 10; concentration, 50 mM), and the incubation medium was changed to 20 mM HEPES-KOH (pH 7.6) immediately before ^{31}P-NMR measurements. ○, cytoplasm; ●, vacuoles

When the cells had been incubated with 10 mM NH_4OH, no red-colored vesicles were observed after the neutral red treatment (Fig. 2c). This observation suggests that the acidic compartments (vacuoles) were neutralized (at least pH changed to above 6.8) by the ammonium accumulated in these organelles during the treatment (Poole and Ohkuma 1981).

Effects of cellular growth phase on the development of acidic compartments

In contrast to the cells at the stationary growth phase (Fig. 1a), an NMR spectrum of intact cells at the logarithmic growth phase shows only one detectable Pi peak (Fig. 1d). According to the cytochemical data stained with neutral red (data not shown), and chloroquine (Fig. 2d), many tiny acidic vesicles were observed in some cells, but their total volumes per cell were much smaller than those in the cells at the stationary phase (Fig. 2b).

Effects of anaerobic treatments on intracellular pH

The treatment with N_2 for 150 min in the dark did not affect the pH values in the cytoplasmic and vacuolar compartments of the cells kept in a mineral medium (Table 1). However, the N_2-bubbling in the presence of 0.2% glucose caused the acidification of cellular pH. In this case, the change in the cytoplasmic pH deduced from the chemical shift of the main Pi signal was much smaller than that in the acidic compartments (Table 1). Cells incubated overnight on ice as pellet showed the same acidification (data not shown).

Effects of the pH of preincubation media on intracellular pH

The algal cells preincubated in 50 mM buffer at different pH under illumination with shaking for 2 h did not cause any pH change in both of the two intracellular compartments (Fig. 3).

Discussion

In the present study, we showed two Pi signals, one represents that at pH 7.1 ± 0.2 and the other at pH 6.3 ± 0.2, in the ^{31}P-NMR spectrum of the cells of *Chlorella vulgaris* 11h at the stationary growth phase (Fig. 1a), and the presence of the organelles whose pH is below 6.8 according to the neutral red staining (Fig. 2a). We further demonstrated that the acidic Pi signal disappeared after the incubation of cells with NH_4OH (Fig. 1c), and this was correlated with the disappearance of acidic organelles by the same treatment (Fig. 2c). We did not detect the acidic Pi signal in *Chlorella* cells at the logarithmic growth phase (Fig. 1d), in which only tiny acidic vesicles were observed by the cytochemical staining (Fig. 2d). Thus the acidic Pi signals in the ^{31}P-NMR spectra were well correlated with the microscopic evidences for acidic compartments (vacuoles). We therefore concluded that Pi at pH 6.3 is located in the vacuoles.

In *Chlorella vulgaris* 11h cells, Mitsumori and Ito (1984) reported that the vacuolar Pi signal was not detectable by ^{31}P-NMR. As observed in this work, the development of vacuoles are strongly affected by the growth stage. It is, therefore, possible that they used the cells in which the vacuoles were not developed well. In this connection, Sianoudis et al. (1984) reported that in the synchronous culture of *Chlorella fusca*, pattern of the two Pi signals on ^{31}P-NMR spectra varied depending on the stage of the cell cycle.

The vacuolar pH in intact cells of *Chlorella vulgaris* 11h is slightly more alkaline than in *Chlorella fusca* (pH 5.6, Sianoudis et al. 1984), in higher plants (pH 5.5, Roberts et al. 1980) and Charophyta (pH 5.25, Mimura and Kirino 1984), but almost the same as in yeasts (pH 6.3–6.4, Nicolay et al. 1982).

Our cytochemical staining methods applied for the first time to the vacuoles in the living unicellular algal cells, could also be applied to the same organelles of *Chlamydomonas reinhardtii* and *Euglena gracilis* (unpublished work). It is easy to observe the morphological changes and developments of vacuoles in intact cells as well as to estimate their internal pH qualitatively. Boller and Wiemken (1986) reviewed evidences indicating that the vacuoles change form and structure during growth and differentiation in tissues of higher plants and in yeast cells. The cytochemical staining technique for vacuoles presented in this paper would serve for the study on their behavior in algal cells under various physiological conditions. It should be noted, however, that the volume of stained spaces does not always represent the actual volume, because of the vacuolating effect of the basic substances added (Ohkuma and Poole 1981).

After anaerobic treatment in the presence of glucose, both cytoplasmic and vacuolar pH became more acidic, and the extent of acidification was larger in the vacuolar pH (Table 1). These results suggest that acid fermentation occurred during the anaerobic treatment of *Chlorella* cells in the presence of glucose. Syrett and Wong (1963) reported that acids produced during the fermentation were lactic, acetic and formic acids in the decreasing order. Kowallik and Gaffron (1976) also reported the excretion of acidic substances from *Chlorella* cells under anaerobic conditions. The intravacuolar accumulation of these acids may occur to keep the homeostasis of pH and metabolite concentration of cytoplasm. In this connection, Huber-Wälchli and Wiemken (1979) reported the accumulation of some excess amount of amino acids in the vacuoles of yeast cells. The acidification of vacuoles was observed only when glucose was added in the media (Table 1). Endogenous fermentation products accumulated would have been too little to affect the pH of

these cells, since the cells starved in K_2SO_4 solution were used in this experiment.

On the other hand, the incubation in acidic media did not cause accumulation of acidic compound in the cytoplasm as well as in the vacuoles (Fig. 3). *Chlorella* cells are also known to be tolerant to environmental pH changes (Lane and Burris 1981).

One might argue that the present results do not reflect the direct effect of external pH, since the cells were suspended in a buffer at pH 7.6 immediately before NMR measurement (see Materials and methods). However, the pH changes in the vacuoles induced by adding NH_4OH under aerobic conditions or glucose under anaerobic conditions were observed in the same buffer solutions (Table 1). Therefore we may conclude that at least the vacuolar pH is not affected by the external one.

In connection with the finding that the vacuoles are more developed in the cells at the stationary growth phase than those in the logarithmic phase, it would be noted that the activities of lysosomal enzymes and turnover of the macromolecules in yeast cells were markedly higher in stationary-phase cells, than in the logarithmically growing cells (Matile et al. 1969). The vacuoles in unicellular algae may play roles as lysosomes and also as "metabolic buffer" of the cytoplasm.

Chloroquine is known as an effective alkalizing reagent for acidic compartments (Poole and Ohkuma 1981). It would be also useful as an inhibitor for characterizing functions and physiological meanings of the acidicity of the vacuoles. It is reported that the reagent inhibited the proteolytic activity in yeast (Lenz and Holzer 1984).

With the respect to the dynamic motion of intracellular vesicles in which neutral red and chloroquine were specifically accumulated (vacuoles), Palevitz and O'Kane (1981) reported the continually moving vacuolar networks in stomatal cells of *Allium*.

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