Phosphorus-31 nuclear magnetic resonance studies of photosynthesizing *Chlorella*

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Phosphorus-31 nuclear magnetic resonance studies of intact *Chlorella* cells under light and dark conditions are described. P_i, ATP, NAD, UDP-glucose and polyphosphate were observed in the cell. The presence of two compartments was postulated from two intracellular P_i signals, whose chemical shift values were dependent on illumination. These two P_i signals were assigned to those in the stroma of chloroplasts and in the cytoplasm based on their response to the light and dark cycle, and to the treatment of cells with 3-(3,4-dichlorophenyl)-1,1-dimethylurea. In the light the chloroplastic pH became more alkaline, while the cytoplasmic pH became more acidic. An increase in ATP was also observed upon illumination.

^{31}P-NMR Chlorella *Intracellular pH* *Chloroplast* *Cytoplasm* *Photophosphorylation*

1. INTRODUCTION

^{31}P-NMR has been successfully applied to study intact living tissues, microorganisms and whole animals or human beings [1,2]. Recently there has been increased interest in the application of NMR to the study of intact plant cells, most of which dealt with non-photosynthetic tissues such as root tips or cultivated cells in the dark [3–5]. However, plant cells are characterized by light-mediated processes such as photosynthesis and photorespiration, which are particularly intriguing subjects to be examined with NMR. There have been several reports of intracellular changes under illumination in photosynthetic bacteria [6,7], blue-green algae [8] or leaf tissue of higher plant [9,10] using ^{31}P-NMR. In these reports only one P_i signal was observed in the cell except that vacuolar P_i was additionally observed in the leaf tissue [10]. Under illumination the intracellular P_i resonance was reported to show shift downfield, indicating an increase in the pH of the compartment, while the vacuolar P_i was insensitive to illumination. P_i signals sensitive to light were assigned to cytoplasmic P_i in photosynthetic bacteria and blue-green algae which have no chloroplasts [6–8]. A light-sensitive P_i signal in the leaf tissue was shown as an extravacuolar P_i and was not identified clearly [10].

In the present work, we report ^{31}P-NMR studies of intact photosynthetic *Chlorella* under light and dark conditions, where two compartments corresponding to the stroma of chloroplasts and the cytoplasm were simultaneously observed for the first time to our knowledge. The pH changes in both compartments along with ATP production in the light will be discussed.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MDP, methylene diphosphonic acid

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2. MATERIALS AND METHODS

2.1. *Chlorella* cells

Chlorella vulgaris was grown autotrophically by bubbling CO_2 gas (CO_2, 5%, air, 95%) under illumination. The composition of the culture medium was 10 mM urea, 10 mM $MgSO_4$, 9.2 mM KH_2PO_4, 0.6 mM K_2HPO_4, 70 μM Fe–EDTA and Arnon

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A-5 micronutrients with the amounts of Mn^{2+} and Cu^{2+} reduced to one-tenth. Cells were harvested 3 days after the inoculation and washed 3 times by the culture medium modified to contain 100 mM KCl, 20 mM Hepes (pH 7.0), and no potassium phosphates or paramagnetic ions. For NMR measurements in the dark 1 ml of packed cells were suspended in an equal volume of the modified culture medium containing 10% D_2O. *Chlorella* cells twice diluted were used for the measurements with an apparatus for in situ illumination.

2.2. NMR measurements

^{31}P-NMR measurements were performed at 161.8 MHz using a JEOL GX-400 spectrometer without proton noise decoupling. A typical spectrum was obtained by accumulating 2500 transients with a recycle time of 0.22 s and a 45° pulse. Chemical shift values were from external methylene diphosphonic acid (MDP) solution sealed in a capillary. Illumination of cells during NMR measurements was performed using a 1 kW xenon lamp as a light source. The light, whose ultraviolet and infrared regions were cut off, was guided to the cell via optical fiber and a quartz rod as described elsewhere [11]. At the upper edge of the sample position the light intensity was $>3 \times 10^5$ lux. Appropriate gas bubbling (10 ml/min) was also employed to maintain adequate cell environments during NMR measurements.

3. RESULTS AND DISCUSSION

When phototrophically grown *Chlorella* cells were quickly prepared within a few tens of minutes of switching the light off, the ^{31}P-NMR spectrum of the cells showed two intracellular P_i signals in addition to some organic phosphates and polyphosphate even in the dark (fig.1A). These two P_i signals came closer and finally became one signal when the cells were aerobically incubated in the dark (fig.1B). This finding suggests the presence of two light-dependent compartments in the cells.

To determine the internal pH of the compartments described above from the chemical shift value of the P_i signal, a pH titration was carried out in the cell suspension in the presence of 100 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) which cancels the transmembrane pH gradient. In the pH range between 5 and 8 intra- and extracellular P_i titrated coincidently. As a result a pH titration curve of P_i in the cell with the pK_a value of 6.9 was obtained, which can be used to calibrate the chemical shift of the intracellular P_i with internal pH.

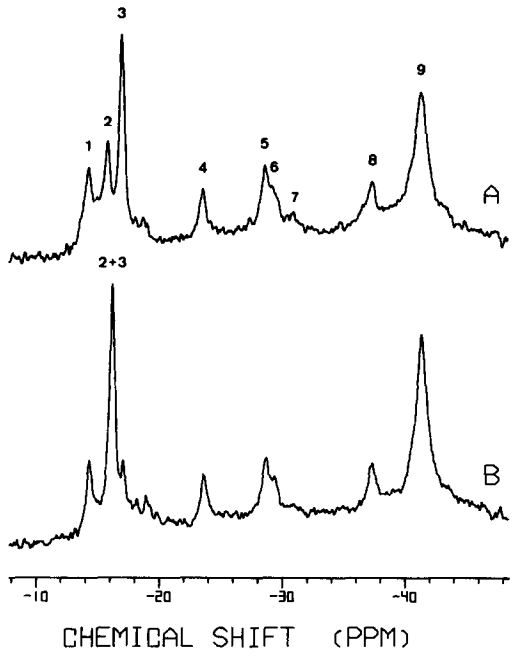

Fig.1. 161.8 MHz ^{31}P-NMR spectra of intact autotrophically grown *Chlorella* cells measured in the dark (A) 27 min and (B) 137 min after light was turned off. Spectral assignments are 1, sugar phosphates; 2, chloroplastic P_i; 3, cytoplasmic P_i; 4, ATPγ; 5, ATPα; 6, NAD + UDP-glucose; 7, UDP-glucose; 8, ATPβ; 9, polyphosphate. Chemical shift values are in ppm from external MDP. Packed *Chlorella* cells (1.0 ml) were suspended in the equal volume of medium containing KCl (100 mM), several mineral nutrients, Hepes (20 mM), and D_2O (10%). The initial pH of the cell suspension was adjusted to 7.0. External P_i had been removed by washing cells with P_i free medium three times. The 2500 transients were accumulated with the recycle time of 0.22 s at 25°C. O_2 bubbling (10 ml/min) was performed to maintain aerobic conditions during NMR measurements.

Based on this calibration the pH values of the two intracellular compartments shown in fig.1A were determined to be 8.1 (lower field P_i signal) and 6.8 (upper field P_i signal), respectively. Fig.2A shows the time dependent pH changes in these two compartments in the dark. When the cells were preincubated in the presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) for 30 min

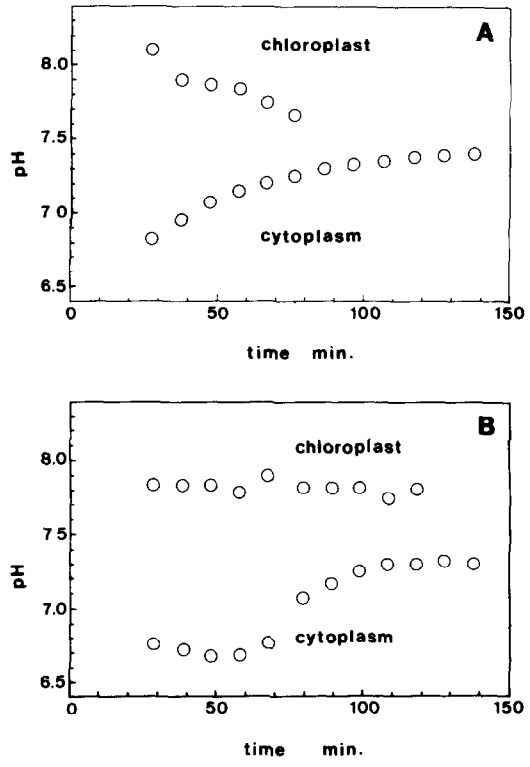

Fig.2. Time course of changes in the chloroplastic and cytoplasmic pH values during dark incubation determined by the chemical shift values of the corresponding P_i peaks. O_2 bubbling (10 ml/min) was performed to maintain aerobic conditions during NMR measurements. Cells were preincubated under illumination for 30 min (A) in the absence, and (B) in the presence of 10 μM DCMU. Incubation time was counted from the time when the light was turned off.

under illumination before harvesting, the internal pH of the compartment which corresponded to the lower field P_i peak decreased from 8.1 to 7.8, and the time dependent change of this peak in the dark disappeared (fig.2B).

The major compartments in intact *Chlorella* cell are cytoplasm, the stroma of chloroplast and the interspace of thylakoid membrane. The interspace of mitochondria and vacuoles were assumed to be negligible in case of *Chlorella* cells. In the light protons are known to move from the stroma of chloroplast to the interspace of thylakoid membrane, which increases the pH of stroma [12]. This change is inhibited by DCMU. Based on the findings described above the compartment which corresponded to the P_i peak at lower field was identified with the stroma of chloroplast. The pH change in the compartment corresponding to the P_i peak at higher field was consistent with that expected in the interspace of thylakoid, whereas the change was not inhibited by DCMU. In addition the interspace of thylakoid occupied only about one-tenth of the stroma in spinach chloroplast [12]. Therefore, this compartment was not thought to be the interspace of thylakoid but rather the cytoplasm.

To verify the changes in the above-mentioned compartments under illumination, measurements were carried out in the light and the dark. The apparatus for in situ illumination was assembled as described in section 2. Fig.3 shows the ^{31}P-NMR spectra of intact *Chlorella* cells under light and dark conditions. Though in the dark only one P_i peak was observed, it split into two when the light was turned on. Intracellular ATP was found to be increased in the light. This increase in ATP was

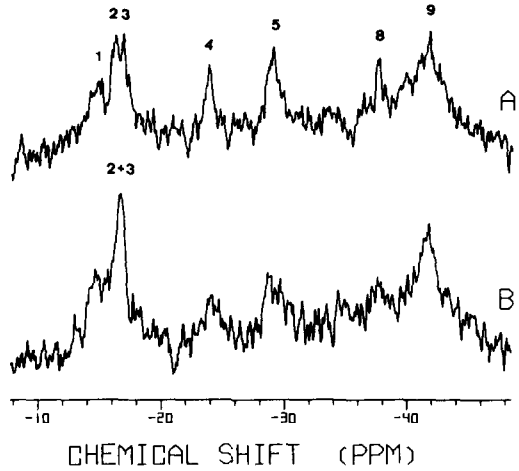

Fig.3. 161.8 MHz ^{31}P-NMR spectra of intact autotrophically grown *Chlorella* cells measured (A) in the light, and (B) in the dark with the light-illuminating apparatus. Spectral assignments are the same as shown in fig.1. Chemical shift values are in ppm from external MDP. The cell suspension was twice as dilute as that used in the measurements shown in fig.1, and the external pH was adjusted to 7.4. The 3750 transients were accumulated with the recycle time of 0.45 s at 25°C. Gas bubbling (CO_2, 5%; N_2, 95%) was performed to supply CO_2 to the cells under anaerobic conditions during NMR measurements.

due to photophosphorylation, since the activity of oxidative phosphorylation would be substantially lowered by bubbling O_2-free gas (CO_2, 5%; N_2, 95%). The pH change of the compartments in the dark and light cycle in the absence and presence of DCMU is shown in fig.4. It was clearly demonstrated that light induced an increase in pH of the stroma of chloroplast, which was inhibited by the presence of DCMU. These findings were consistent with changes in the intracellular pH under illumination reported with photosynthetic bacteria [6], blue-green algae [8] and leaf discs of wheat [10]. However, this is the first report to unequivocally identify the P_i derived from the stroma of chloroplast in intact cells.

According to the chemiosmotic theory, the electrochemical potential gradient of protons across the thylakoid membrane is utilized to synthesize ATP by ATPase [13]. The pH change in stroma of chloroplast under illumination observed in the present work represents one side of the proton gradient. Unfortunately the other side, the interspace of thylakoid, could not be observed at present presumably due to its small volume fraction compared with that of stroma [12]. As mentioned earlier, the increase in ATP under illumination was also observed at the same time. In the present work it is shown that ^{31}P-NMR will be a potent method to elucidate the bioenergetics in photophosphorylation as well as in oxydative phosphorylation [14].

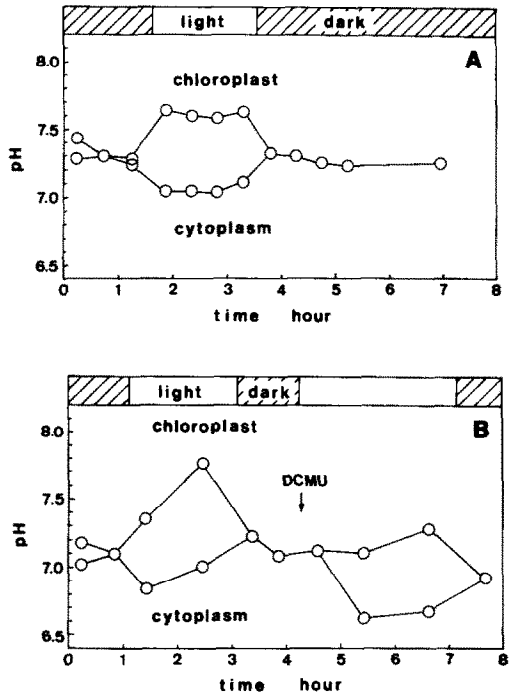

Fig.4. Time course of changes in chloroplastic and cytoplasmic pH values in the light and dark cycle determined by the chemical shift values of corresponding P_i peaks. In (B) DCMU was added to give the final concentration of 50 μM at the time indicated by a vertical arrow. CO_2 was supplied under anaerobic conditions with gas bubbling (CO_2, 5%; N_2, 95%) during NMR measurements.

On the other hand, the decrease in cytoplasmic pH was induced by illumination, and was not inhibited by DCMU (fig.2,4). The reason why the cytoplasmic pH decreased in the light has not been elucidated so far. Proton extrusion from chloroplast in the light was reported in case of isolated chloroplast [12,15], but it may be insufficient to explain the pH change in cytoplasm by as much as 0.5 pH unit found in the present work. Another possibility to cause the acidification of cytoplasm is the accumulation of inorganic carbon ($CO_2 + HCO_3^-$) in the cell under light condition, since the accumulation was presumed to be accompanied with either the influx of H^+, efflux of OH^- or both [16]. The phenomenon was widely observed in various kinds of green algae, blue-green algae and aquatic angiosperm [16]. In a cyanobacterium the reverse process of the accumulation, CO_2 burst in the dark, was reported to be insensitive to DCMU [17]. Now further experiments to make clear the mechanism for the acidification of the cytoplasmic pH under illumination are in progress.

It is interesting that the pH of cytoplasm as well as stroma of chloroplast depends on light. The enzymes located in chloroplast such as ribulose-1,5-bisphosphate carboxylase and fructose bisphosphatase are activated by an increase in stromal pH along with the intake of Mg^{2+} induced in the light [18,19]. In cytoplasm the decrease in pH caused in the light might be a regulating factor of carbon metabolism in a similar way. For example glycolysis, which functions in cytoplasm, is known to be slowed down at low pH through key enzymes such as phosphofructokinase [20]. This change, if in fact it occurred, is consistent with the general concept that the flow of fixed carbon in the light is directed toward sucrose synthesis rather than toward glycolysis.

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