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pH-dependent regulation of carbonic anhydrase induction and change in photosynthesis during adaptation of *Chlorella* cells to low CO_2

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Induction of carbonic anhydrase (CA) activity was pH-dependent when *Chlorella* grown under 3% CO_2 in air was transferred to low CO_2 conditions (ordinary air) at various pHs. Optimum pH for CA induction was 8.0 in *C. ellipsoidea* C-27, which has both intracellular and extracellular CAs, and 7.0 to 8.0 in *C. regularis* which has CA mostly on the cell surface. Below pH 5.5, CA induction was suppressed in both species even under low CO_2 conditions. As photosynthetic O_2 evolution in *C. ellipsoidea* C-27 adapted to low CO_2 for 3 h was independent of external pH when measured under the condition used for adaptation, the pH-dependency of CA induction cannot be attributed to that of the photosynthetic activity.

The rate of photosynthesis was kept constant at both pH 8.0 and 5.5 during adaptation to low CO_2. However, the rate measured with ethoxyzolamide (EZA), an inhibitor of CA, decreased gradually at both pHs. The suppression by EZA was not observed in the presence of 10 mM $NaHCO_3$. Rate of photosynthesis under CO_2-limiting conditions in cells adapted to low CO_2 with cycloheximide decreased gradually at both pH 8.0 and 5.5.

These results suggest that high rate of photosynthesis under CO_2-limiting conditions in cells adapted to low CO_2 is due to the function of carbonic anhydrase at high pH and due to an EZA-sensitive protein factor, which may enhance CO_2 transport, at low pH.

Key Index Words: carbonic anhydrase—Chlorella ellipsoidea—Chlorella regularis—*CO_2 acquisition—enzyme induction—low-CO_2 adaptation—pH effect—photosynthesis.*

Algal cells grown in ordinary air (low-CO_2 cells) exhibit higher affinity for CO_2 in photosynthesis and higher activity of carbonic anhydrase (CA) than those grown in CO_2-enriched air (high-CO_2 cells) (see review by Raven 1984, Aizawa and Miyachi 1986, Badger 1987). Those changes induced during adaptation to low CO_2 have been reported to be regulated by several environmental factors. For example, light plays an important role in CA induction and its effect is different depending on algal species. Namely, CA induction in *Chlamydomonas reinhardtii*, which has mainly extracellular CA, showed a requirement of both high energy of light for photosynthesis and low energy of blue light as a photosignal (Kimplel *et al.* 1983, Dionisio *et al.* 1989a, b, 1990). However, only photosignal is essential for CA induction in *Chlorella vulgaris* 11 h, which has only intracellular CA (Shiraiwa *et al.* 1981, Shiraiwa and Miyachi 1983) and in *Chlorella regularis*, which has mostly extracellular CA (Umino *et al.* 1991). Effect of other factors such as CO_2 concentration (Shiraiwa and Miyachi 1985), temperature (Shiraiwa and Miyachi 1985) and O_2 concentration (Shiraiwa *et al.* 1988) on CA induction were examined mainly in *Chlorella vulgaris* 11 h, but few works in other algae.

Recently, pH was shown to be an important factor regulating CA induction in *Chlamydomonas*. Patel and Merrett (1986)

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Abbreviations: CA, carbonic anhydrase; DIC, dissolved inorganic carbon; EZA, ethoxyzolamide; CHI, cycloheximide; high-CO_2 cells, algal cells grown in air enriched with 3% CO_2; low-CO_2 cells, algal cells grown in air; pcv, packed cell volume.

showed that CA induction during adaptation of high-CO_2 cells to air was enhanced concomitantly with pH, although how the induction of CA is controlled by pH is not elucidated yet. As dissociation of DIC is strongly dependent on pH of the medium, phenomena induced by a change in DIC concentration, such as CA induction and change in an affinity of photosynthesis, may be probable to be affected directly and/or indirectly by change in pH. Response of the induction of CAs with different locations in a cell to external pH is also interesting to be compared.

In the present study, we therefore investigated the effect of pH on CA induction within a wide range of pH, from 3 to 9, in two species of *Chlorella* which have different CA localization. Effect of pH on CO_2 acquisition in photosynthesis during adaptation of high-CO_2 cells to low CO_2 conditions was also tested.

Materials and Methods

Algal materials and culture—Chlorella ellipsoidea Gerneck (IAM C-27) was obtained by courtesy of Prof. T. Hirokawa of Niigata University. *Chlorella regularis* (Endo *et al.* 1974) was a kind gift of Prof. S. Miyachi of University of Tokyo. These algae were grown autotrophically in a flat oblong glass vessel containing *ca.* 1.3 liter of the inorganic MC medium (Watanabe 1960). The suspensions were continuously aerated with ordinary air enriched with 3% CO_2 to obtain cells adapted to high CO_2 (high-CO_2 cells). To obtain air-adapted cells (low-CO_2 cells), the cells harvested by centrifugation were suepended in an appropriate buffer at a density of 3 m*l* pcv·liter^{-1}, and then transferred to air. The algal suspension was continuously illuminated by 200 W-incandescent reflector lamp (Toshiba, Tokyo) at 1.2 kW·m^{-2} (16 klux). The temperature during the growth and the adaptation to air was kept under the optimum conditions for photosynthesis and growth in each alga, namely at 25°C in *Chlorella ellipsoidea* and at 30°C in *Chlorella regularis*.

Determination of photosynthetic O_2 evolution—Algal suspension (5 ml) of *Chlorella ellipsoidea* harvested from the culture was immediately transferred into a water-jacketed transparent glass-cylinder equipped with a Clark-type oxygen probe (Rank Brothers, London). After 1-min incubation in the dark, photosynthesis was initiated by illumination by a tungsten projector lamp at 1.4 kW·m^{-2} (18 klux), and change in O_2 concentration in the medium was continuously measured by the O_2-electrode. The temperature was kept at 25°C.

Carbonic anhydrase assay—Enzyme assay was carried out according to the method of Wilbur and Anderson (1948). To measure CA activity localized on the cell surface of intact cells (E), 2 m*l* of CO_2-saturated water was added to 3 m*l* of 20 mM (final concentration, 12 mM) sodium veronal buffer (pH 8.3) containing 100 μl of the suspension of intact cells suspended in 100 mM Tris-H_2SO_4 buffer (pH 8.3). The time required for pH change from 8.3 to 7.3 was measured at 2°C. The reaction mixture was continuously stirred by a magnetic stirrer. To determine CA activity in cell homogenates (H), 100 μl of homogenates obtained by disruption of algal cells suspended in 100 mM Tris-H_2SO_4 buffer (pH 8.3) with a French Pressure Cell (Ohtake Seisakusho, Tokyo) at 147 MPa (1,500 kg·cm^{-2}) was used for the CA assay. Enzyme units were calculated using the following equation:

$$\text{units} = t_b \cdot t_e^{-1} - 1$$

where t_b and t_e represent the time (seconds) needed for the pH change with or without sample, respectively. Internal CA activity (I) was calculated by H−E.

Results

Effect of pH on the increase in CA activity during adaptation of high-CO_2 cells to low CO_2 for 3 h was tested in *Chlorella ellipsoidea* C-27 and *Chlorella regularis* (Fig. 1). CA activity detected both inside the cells and on the cell surface was almost equal in *Chlorella ellipsoidea* C-27, whereas more than 97% of CA was located on the cell surface in *Chlorella regularis*. Both intra- and extracellular CA ac-

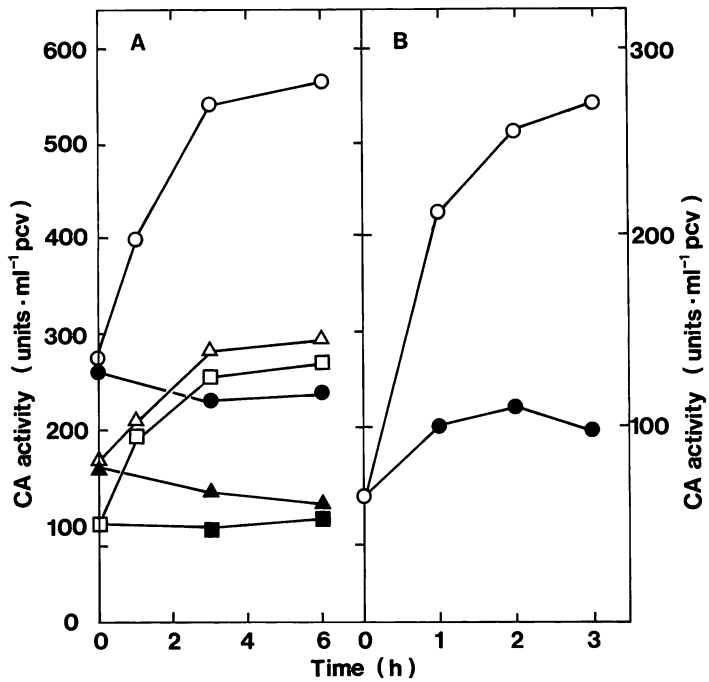

Fig. 1. Time course of CA induction in *Chlorella* after the transfer of high-CO_2 cells to air-level CO_2. A: *Chlorella ellipsoidea* C-27. Open and closed marks, in cells adapted to low CO_2 at pH 8.0 and pH 3.0, respectively. Circles, CA activity in cell homogenate; triangles, extracelular CA activity; squares, intracellular CA activity. B: *Chlorella regularis*. Open and closed circles, extracellular CA activity in cells adapted to low CO_2 at pH 8.0 and pH 5.5, respectively.

tivities of *Chlorella ellipsoidea* took a paralel time course and increased 2-3 times during 3-h adaptation to air at pH 8.0, but no increase in any CA activities was observed at pH 3.0 (Fig. 1A). Extracellular CA activity of *Chlorella regularis* also increased 3 times during 3-h adaptation to air at pH 8.0, but only slight increase was observed at pH 5.5 (Fig. 1B). Optimum pH for the increase in intra- and extracellular CA activities was 8.0 in *Chlorella ellipsoidea* C-27 (Fig. 2A). Intracellular and extracellular CA activities were similarly changed depending on pH. The optimum pH for the increase in external CA in *Chlorella regularis* was 7-8 (Fig. 2B).

In *Chlorella ellipsoidea* cells adapted to low CO_2 at various pHs from 5.5 to 9.0 for 3 h, photosynthetic activity was measured immediately after the transfer of algal suspension from the culture to the reaction vessel. The activity measured without any additives showed no marked variation between pH 5.5 and 9.0. However, the activity measured with 10 mM $NaHCO_3$ clearly exhibited pH-dependence with a peak at pH 8.0 (Fig. 3). The rate of photosynthetic O_2 evolution was suppressed at 10 mM $NaHCO_3$ below pH 6.5, but enhanced above the pH.

When high-CO_2 cells of *Chlorella ellipsoidea* C-27 were transferred to air at pH 8.0 and 5.5, the rates of photosynthesis were almost constant during the adaptation to air at pH 8.0 and 5.5 (Fig. 4). At pH 8.0, the rate was gradually decreased by the addition of 0.1 mM EZA, a membrane-permeable inhibitor of CA, to reach about one-half value of the control. The suppression was recovered by the addition of 10 mM $NaHCO_3$. At pH 5.5, the rate of photosynthesis was strongly decreased at 0.01 mM EZA, but the rate was recovered at 1 mM $NaHCO_3$. The recovery rated almost 100% at the beginning of air-adaptation, but almost linearly decreased by 50% of the value in control for 200 min

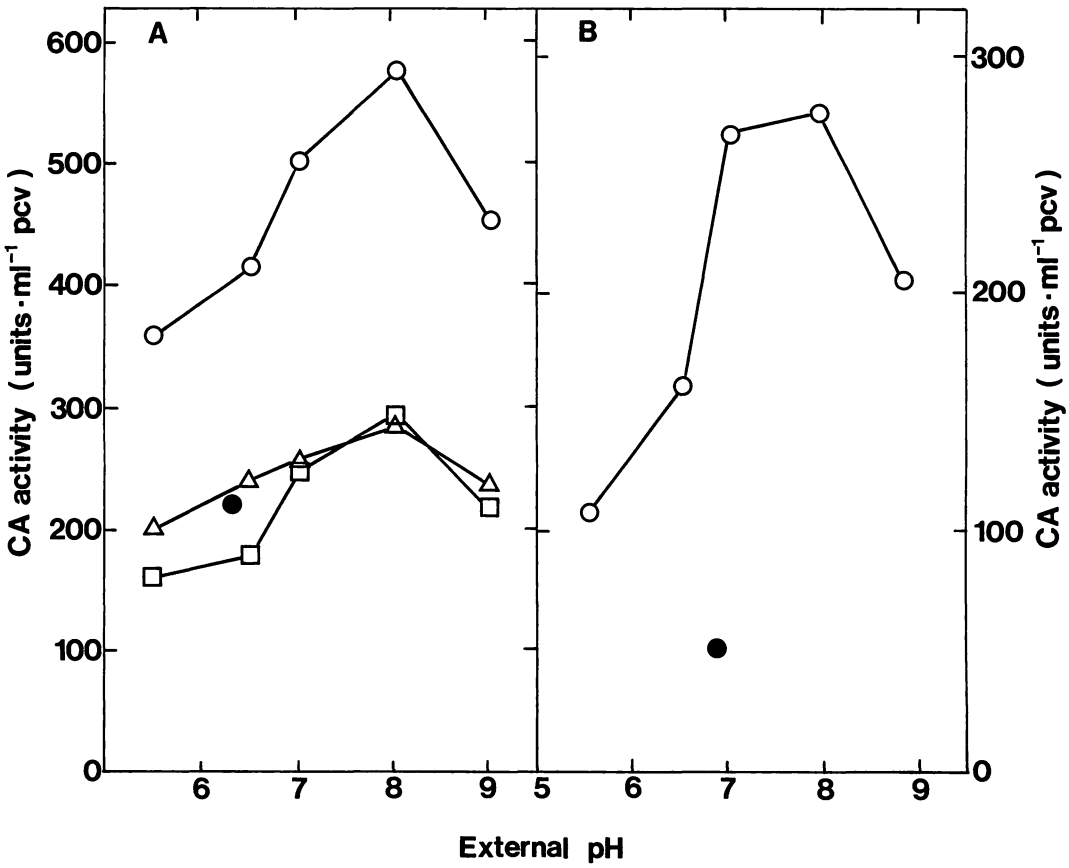

Fig. 2. pH-dependent curves of CA induction in *Chlorella* when high-CO_2 cells were adapted to air-level CO_2 for 3 h. A: *Chlorella ellipsoidea* C-27. Circles, CA activity in cell homogenate; triangles, extracellular CA activity; squares, intracellular CA activity; closed circles, CA activity in the cell homogenate of high-CO_2 cells. pH was kept nearly constant at respective pH by 20 mM MES-Tris buffer containing 1/20 concentration of the culture medium during the adaptation to air. B: *Chlorella regularis*. Open circles, extracellular CA activity in low-CO_2 cells; closed circles, extracellular CA activity in high-CO_2 cells. pH was constantly maintained at respective pH by 50 mM MES-NaOH buffer below pH 6.5 and by 50 mM Tris-H_2SO_4 buffer above pH 7. Both buffer contained 1/20 concentration of the culture medium.

(Fig. 4C). The rate of photosynthesis in control decreased with time when high-CO_2 cells were transferred into air with cycloheximide (CHI), an inhibitor of translation of protein synthesis on 80 S ribosomes (Fig. 4B, D). The rate was hardly affected by EZA at pH 8.0 (Fig. 4B), but strongly limited at pH 5.5 for about 2 h after the start of the adaptation (Fig. 4D). Photosynthesis under CO_2-saturating conditions in CHI-treated cells did not change during the adaptation to low CO_2 at both pH 8.0 and 5.5 (Fig. 4B, D). 0.1 mM EZA was inhibitory to the maximum photosynthesis at pH 5.5, but not at pH 8.0 (unpublished data and Fig. 4A).

Discussion

CA induction during the adaptation of high-CO_2 cells to low CO_2 preferred alkaline pH rather than acidic pH where it was strongly suppressed (Fig. 1). The result is similar to that observed in *Chlamydomonas* (Patel and Merrett 1986). The optimum pH for CA induction was around 8 in both *Chlorella ellipsoidea* and *Chlorella regularis* which have different localization of CA (Fig. 2). Optimum pH for photosynthesis under CO_2-saturating

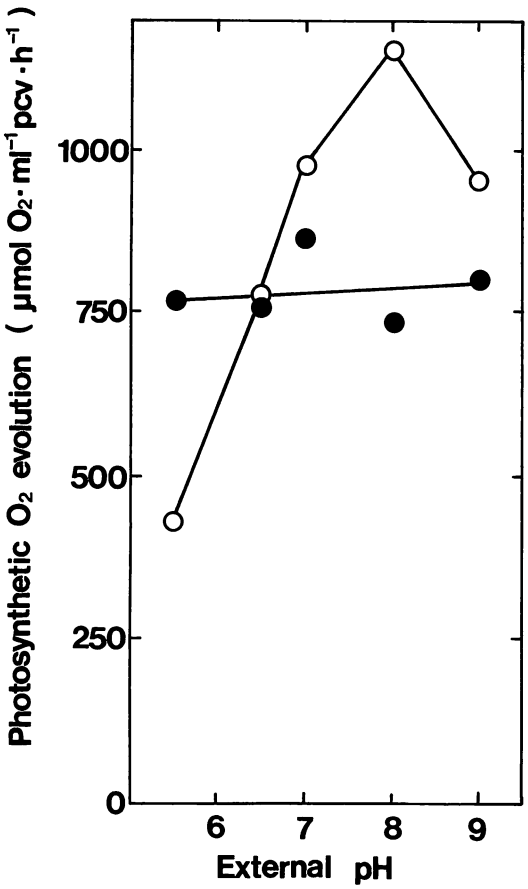

Fig. 3. Effect of pH during adaptation on photosynthesis in *Chlorella ellipsoidea* C-27 adapted to air-level CO_2 for 3 h. Algal cells were adapted to air at various pHs from 5.5 to 9.0 for 3 h, and thereafter the suspension was transferred to the vessel equipped with O_2-electrode. After 1-min incubation in the dark, the rate of photosynthetic O_2 evolution was determined with or without addition of 10 mM $NaHCO_3$. Open and closed circles, rates of photosynthetic O_2 evolution measured with or without the addition of 10 mM $NaHCO_3$, respectively.

conditions was also 8 in both *Chlorella ellipsoidea* (Fig. 3) and *Chlorella regularis* (data not shown). As photosynthesis measured under CO_2-limiting conditions in cells adapted to air for 3 h was similar among various pHs (Fig. 3), difference in CA activity induced at various pHs would be independent of that in photosynthetic activity.

For it has been reported that internal pH was not so strictly affected by changes in external pH in *Chlorella* (Tsuzuki *et al.* 1985), change in CA induction at various pHs could be due to effect of changes in external pH rather than internal one. Active DIC absorbed by cells for photosynthesis in both high- and low-CO_2 cells of *Chlorella ellipsoidea* (Nara *et al.* 1990) and *Chlorella regularis* (Satoh and Shiraiwa, unpublished) is free CO_2, not HCO_3^-. The concentration of free CO_2 dissolved in the culture medium may be equilibrated with that of atmospheric CO_2 because of strong aeration. Therefore, the concentrations are thought to be same among various pHs because the solubility of CO_2 is not affected by pH. These things also suggest that DIC acquisition and CA induction are controlled mainly by changes in pH of the medium.

When high-CO_2 cells were transferred to low CO_2, photosynthesis can be limited by CO_2 transfer from the medium to the site of CO_2 fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase. Under the conditions, physiological basis of adaptation to low CO_2 conditions seems to vary depending on pH. The photosynthetic activity of the cells adapted at pH 8.0 was sensitive to EZA, indicating that CA induction is the major strategy of adaptation to low CO_2 at this pH. The data from the experiments with cycloheximide (Fig. 4A, B) are in fair agreement with this. On the other hand, the adaptation to low CO_2 at pH 5.5 is assumed not to be achieved by the induction of CA, because CA is not functional at acidic pH where DIC mostly exists in the form of free CO_2, an active species of DIC absorbed by *Chlorella* (Nara *et al.* 1990). A reason for inhibition of photosynthesis by EZA at pH 5.5 (Fig. 4C, D) is not elucidated yet. A possible speculation may be the inhibitory effect of EZA on the membrane transport of DIC, since EZA affected the membrane permeability to glycolate in *Chlorella vulgaris* (Shiraiwa and Schmid 1986). Notable in this respect is the fact that the adaptation to low CO_2 at pH 5.5 is sensitive to cycloheximide, as is at alkaline pHs (Fig. 4). This indicates that protein(s) other than CA has to be synthesized for the adaptation, in order to enhance DIC utilization at low pH.

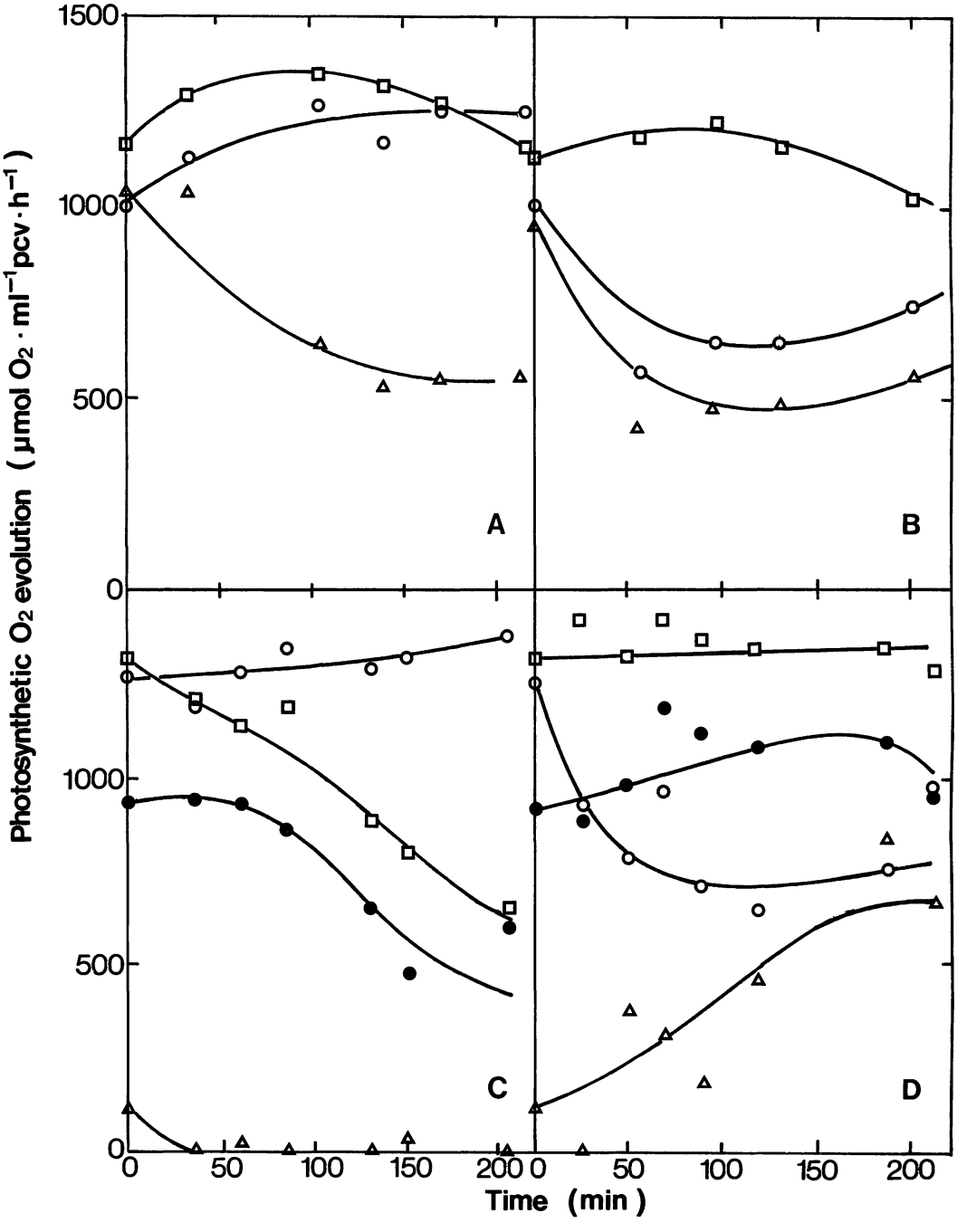

Fig. 4. Change in the rate of photosynthetic O_2 evolution in *Chlorella ellipsoidea* C-27 after the transfer of high-CO_2 cells to air-level CO_2. A and B: Cells adapted to air at pH 8.0 in the absence and presence of 17.8 μM cycloheximide, respectively. Circles, control; triangles, +0.1 mM EZA; squares, +0.1 mM EZA+10 mM $NaHCO_3$. C and D: Cells adapted to air at pH 5.5 in the absence and presence of 17.8 μM cycloheximide, respectively. circles, control; triangles, +0.01 mM EZA; squares, +0.01 mM EZA+1 mM $NaHCO_3$; closed circles, +0.01 mM EZA+10 mM $NaHCO_3$. Buffers used at respective pHs were the same ones as in Fig. 2.

As shown in Fig. 3, photosynthesis in low-CO_2 cells of *Chlorella ellipsoidea* under CO_2-saturated conditions was several times higher at pH 8.0 than pH 5.5, although that under CO_2-limiting conditions was almost same at various pHs. Photosynthesis at pH 5.5 was diminished by the addition of high concentration of DIC. The reason for the suppression is still unclear. One possibility, as assumed by Hogetsu and Miyachi (1979), is a strong drop of internal pH caused by absorbing huge amount of CO_2 and the subsequent conversion to HCO_3^- and H^+. These results in *Chlorella* are inconsistent with those, reported in *Chlamydomonas* by Patel and Merrett (1986), showing that photosynthesis under CO_2-saturated conditions at pH 5.5 was three times higher than that at pH 7.5 in both high- and low-CO_2 cells. As the concentration of DIC accumulated in the cells was two times higher at pH 7.5 than pH 5.5 in low-CO_2 cells, but was independent of pH in high-CO_2 cells, it is considered that photosynthetic activity is dependent on external pH proper, but independent of internal DIC accumulated. The mechanism how external pH affects the CA induction and the change in photosynthetic CO_2 fixation during adaptation to low CO_2, and the reason why pH-dependency of photosynthesis is different between *Chlorella* and *Chlamydomonas* remains to be elucidated.

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白岩善博・横山真也・佐藤　朗：クロレラの低 CO_2 条件への適応に伴うカルボニックアンヒドラーゼ誘導と光合成活性変動の pH による調節

カルボニックアンヒドラーゼ(CA)の局在性が異なる二種の単細胞緑藻 *Chlorella ellipsoidea* C-27 および *Chlorella regularis* を用いて低 CO_2 条件への適応に伴う CA 誘導および光合成の CO_2 に対する親和性の変動に及ぼす pH の影響を調べた。CA 誘導の至適 pH は藻種および CA の局在性に関わらず pH 8 付近であり，pH 5.5 以下では CA の誘導は認められなかった。高（3％）CO_2 条件に適応した *C. ellipsoidea* を低（0.03％）CO_2 条件に移した場合，CO_2 律速条件における光合成活性は pH 5.5 および8.0のいずれの pH でも変動しなかった。しかし，CA 阻害剤であるエトキシゾルアミド添加条件下では CO_2 律速条件下での光合成は時間と共に減少した。また，いづれの pH でも，低 CO_2 への適応時にシクロヘキシミドを添加すると，光合成の CO_2 に対する親和性の増大が阻害された。以上の結果より，これらの藻種が，アルカリ域では CA を誘導することにより，また，酸性域では CO_2 利用を促進する CA 以外のタンパク性因子を誘導することにより CO_2 律速条件下での光合成活性を維持していることが示唆された。（950-21 新潟市五十嵐二の町8050　新潟大学理学部生物学科）