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A Mutant of *Synechococcus* PCC7942 Incapable of Adapting to Low CO_2 Concentration[1]

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ABSTRACT

Some properties of a mutant (RK1) of *Synechococcus* PCC7942, which requires high CO_2 for growth, are described. The photosynthetic affinity for inorganic carbon (C_i) in RK1 was about 40 times lower than that in the wild type (WT) when grown at 3% CO_2 (H-cells) and did not change during 10 hours of exposure to low CO_2 (air containing 0.04% CO_2). The gas exchange of WT and RK1 cells was measured using an open gas-analysis system. All the measurements were performed at a CO_2 concentration of 400 microliters per liter under the conditions where photosynthetic CO_2 fixation is inhibited. When the suspension of H-cells of WT or RK1 was illuminated, the rate of CO_2 influx from the gas phase into the suspension was low and addition of carbonic anhydrase during illumination released only a small amount of CO_2 from the medium into the gas phase. The rate of CO_2 influx and the amount of CO_2 released by carbonic anhydrase were increased in WT during low CO_2 adaptation. These changes did not occur in RK1 during exposure to low CO_2. Cytoplasmic membrane from H-cells of WT or RK1 contained small amount of 42-kilodalton polypeptide. Exposure of RK1 to low CO_2 did not have significant effect on the amount of 42-kilodalton polypeptide, while the same treatment on WT resulted in a large increase of this polypeptide. The RK1 mutant appears to be defective in its ability to utilize the intracellular C_i pool for photosynthesis and also to transmit a low CO_2 signal for inducing the functional and compositional changes observed in WT during low CO_2 adaptation.

Exposure of H-cells[2] of cyanobacteria to low CO_2 conditions increases their C_i-transporting capability and photosynthetic affinity to C_i (3, 6–8, 11, 12). A 42-kD polypeptide is synthesized in the cytoplasmic membrane of *Synechococcus* PCC7942 or PCC6301 during the adaptation process (8, 11, 12, 14). The antibody against this polypeptide cross-reacted with a 45-kD polypeptide in the cytoplasmic membrane of low CO_2-grown cells of *Synechocystis* PCC6803 (13). The results indicated that the synthesis of these polypeptides is one of the features which characterize the adaptation to low CO_2.

The molecular mechanism of C_i uptake and the process of adaptation is poorly understood. Mutants defective in the C_i-transporting system or in their capability of adapting to low CO_2 level are thus highly desirable. Marcus *et al.* (5) have isolated a mutant of *Synechococcus* PCC7942 which requires high CO_2 for growth. The mutant (E_1) was able to adapt to low CO_2 as indicated by the large increases in its ability to accumulate C_i internally and in the amount of the 42-kD polypeptide during exposure to low CO_2 (14). The E_1 mutant appears to be defective in its ability to utilize the intracellular C_i pool for photosynthesis (5). The present study concerns with a mutant (RK1) of *Synechococcus* PCC7942 isolated in our laboratory which requires high CO_2 for growth.

It was shown previously that illumination of the suspension of low CO_2-adapted *Synechococcus* PCC7942 cells produced nonequilibrium between CO_2 and HCO_3^- in the medium, with the concentration of HCO_3^- being higher than that expected under equilibrium conditions (9). Abolishing the nonequilibrium by CA released CO_2 from the medium into the gas phase. It was inferred that the nonequilibrium was produced as a result of CO_2 influx and HCO_3^- efflux (9). We examined in this study the effect of CA on the patterns of CO_2 exchange by WT and RK1 after various periods of exposure to low CO_2. The effect of CA was insignificant in H-cells of WT or RK1 and became pronounced in WT as the adaptation to low CO_2 proceeded. This paper describes the changes in the photosynthetic affinity for C_i, the gas-exchange characteristics, and in the content of the 42-kD polypeptide in WT during low CO_2 adaptation plus demonstrating the lack of these changes in the RK1 mutant exposed to low CO_2.

MATERIALS AND METHODS

Isolation of Mutants. Cells of *Synechococcus* PCC7942 (*Anacystis nidulans* R2) were grown at 34°C in BG11 medium (15) supplemented with 10 mM Hepes-NaOH buffer (pH 7.5), under aeration with 3% CO_2 in air. Continuous illumination was provided by tungsten lamps at 120 μmol PAR/m^2s. Mutants were isolated following mutagenesis with MNNG essentially as described in Herdman and Carr (2). H-cells of WT in a logarithmic phase of growth were treated with MNNG (40 μg/ml) for 20 min in the light, washed twice, and then grown for 2 d in the medium containing ampicillin (100 μg/ml) under aeration with air (0.04% CO_2). The cells were washed twice and then grown in 10 test tubes, each containing 50 ml of the growth medium, under aeration with 3% CO_2 in air. Cells from each tube were plated on an agar plate containing the growth medium and incubated in the light under 3% CO_2 until colonies were formed. Each colony was plated on a pair of agar plates; one incubated under air and the other under 3% CO_2. More than 50% of the colonies did not grow under air. We selected 10 mutants, one from the culture in each test tube. One of these mutants, RK1, described in this paper was very stable with the reversion frequency of less than 10^{-9}.

Gas Exchange Measurements. The mutant and WT cells of

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[2] Abbreviations: H-cells, high CO_2-grown cells; L-cells, cells exposed to low CO_2 for 10 h; C_i, inorganic carbon; CA, carbonic anhydrase; IAc, iodoacetamide; WT, wild type; CBB, Coomassie brilliant blue; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Synechococcus were grown as described above. Cell cultures at a late logarithmic phase of growth were aerated with air for various periods under the same light conditions. Cells harvested by centrifugation (3000*g*, 5 min) were suspended in 20 mM Hepes-NaOH buffer (pH 7.0) containing 15 mM NaCl at a Chl concentration of 4 to 6 μg/ml and placed in a reaction vessel (8). The gas exchange of the cells in the reaction vessel was measured at 30°C using an open gas-analysis system previously described (8) under the conditions where photosynthetic CO_2 fixation is completely inhibited. N_2 containing 2% O_2 and 400 μl CO_2/L was bubbled into the cell suspension in the reaction vessel and the concentration of CO_2 in the exchanged gas was measured with an infra-red CO_2 analyzer (model ZAP, Fuji Electric Co., Tokyo).

Electrophoresis and Immunostaining. Cytoplasmic membranes were prepared as described in Omata and Ogawa (12). The 42-kD polypeptide of low CO_2-adapted cells of in the cytoplasmic membrane of *Synechococcus* PCC7942 was purified by two cycles of SDS-PAGE and was used for raising the antibody in rabbit. IgG fractions were obtained according to Chua *et al.* (1). SDS-PAGE was performed in the buffer system of Laemmli (4). Polypeptides were electrotransferred to nitrocellulose and reacted with the IgG fractions. Goat anti-rabbit IgG/alkaline phosphatase conjugate was used as the second antibody to detect the reacting polypeptide.

Other Measurements. Growth curves were determined from the rise in the *A* at 750 nm measured using a Shimadzu Recording Spectrophotometer (UV-200). Pigments in the cells were extracted by methanol and Chl in the extract was determined according to Ogawa and Shibata (10).

RESULTS

Photosynthetic Affinity for Extracellular C_i. Under high CO_2 (3%) the growth rate of RK1 was similar to that of WT (Fig. 1). RK1 did not grow under low CO_2 (0.04%). The growth rate of WT under low CO_2 was about one-fifth of that under high CO_2. In RK1, apparent photosynthetic affinity for extracellular C_i was about 40 times lower than that of WT; the apparent $K_m(C_i)$ values for photosynthesis were 1.8 mM and 44 μM for H-cells of RK1 and WT, respectively (Fig. 2). Thus the inability of RK1 to grow at air level of CO_2 might be due to the very high $K_m(C_i)$. In WT, L-cells had much smaller $K_m(C_i)$ value than H-cells. In contrast, the $K_m(C_i)$ value for H-cells and L-cells of RK1 was similar. It is not clear why WT grows slowly under low CO_2 in spite of its low $K_m(C_i)$ value.

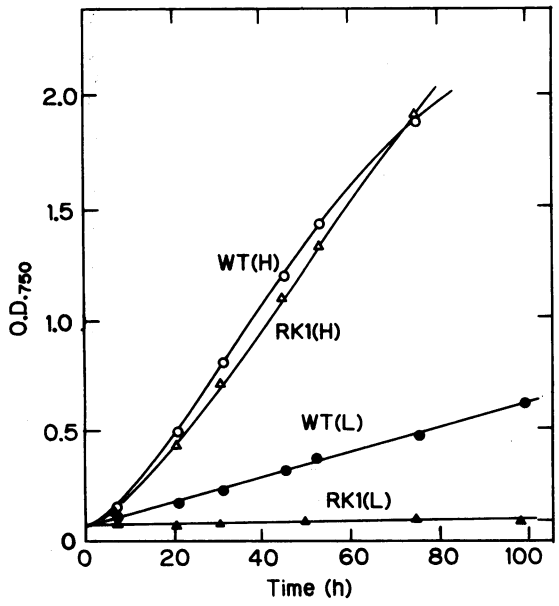

FIG. 1. Growth curves of RK1 and WT of *Synechococcus* PCC7942 at high (H, 3% v/v) and low (L, 0.04% v/v) CO_2 in air.

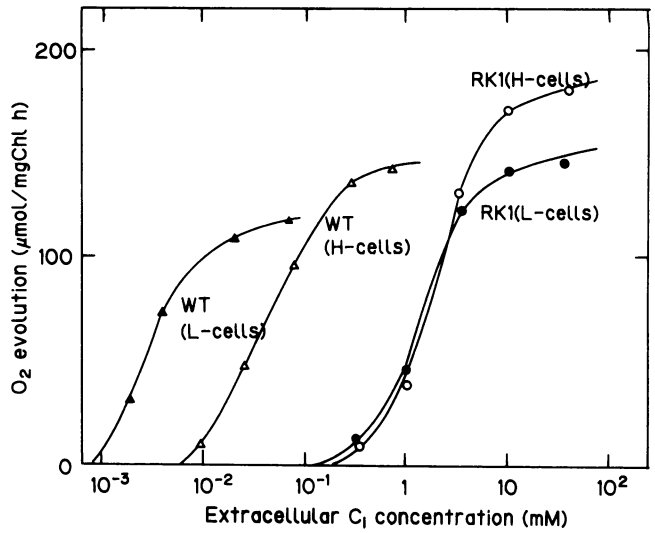

FIG. 2. The rate of photosynthetic O_2 evolution in H-cells and L-cells of RK1 and WT as a function of the extracellular C_i level. Cells were suspended in 20 mM Hepes-NaOH (pH 7.0) containing 15 mM NaCl. Light intensity was 2100 μmol PAR/m^2·s.

CO_2 Exchange Characteristics. The CO_2 exchange patterns of RK1 and WT before and after exposure to low CO_2 (10 h) are shown in Figure 3. RK1 does not perform CO_2 dependent photosynthetic O_2 evolution under the CO_2 concentration (400 μl/L) used in the gas-exchange measurements and photosynthetic CO_2 fixation in WT was inhibited by IAc. Therefore, the CO_2 exchange patterns shown in this figure are independent of photosynthetic CO_2 fixation. IAc had no effect on the CO_2 exchange profiles of RK1. When cells in a reaction vessel were illuminated, there was an influx of CO_2 from the gas phase into the cell suspension (Fig. 3). The maximal rate of CO_2 influx (M in Fig. 3D) was low in H-cells either of RK1 (Fig. 3A) or WT (Fig. 3C). Exposure of RK1 to low CO_2 did not have significant effect on the M (Fig. 3B) while the same treatment on WT resulted in a large increase in M (Fig. 3D).

During about 4 min of illumination, the rate of CO_2 influx decreased slowly to zero (Fig. 3, A–D). When CA was added under these conditions to the suspension of L-cells of WT, more than 80% of the CO_2 taken up during illumination was released into the gas phase (Fig. 3D), being consistent with the previous observation (9). CA did not have any effect when added to the cell suspension in the dark. Thus, a reaction driven by light produced nonequilibrium between CO_2 and HCO_3^- in the medium, with the concentration of HCO_3^- being higher than that expected under equilibrium conditions. The effect of CA was much less in H-cells of RK1 (Fig. 3A) or WT (Fig. 3C) and was negligibly small in L-cells of RK1 (Fig. 3B). The amount of CO_2 evolved by addition of CA, as indicated by CO_2(CA) in Figure 3D, would be equal to the amount of HCO_3^- in the medium in excess of that expected under equilibrium conditions. Thus, in H-cells of WT and RK1 or in L-cells of RK1, CO_2 and HCO_3^- in the suspending medium were nearly or in equilibrium even in the light.

The C_i accumulated within the cells in the light is extruded after darkening. The extrusion is observed as post-illumination CO_2 burst (the right side of the curves in Fig. 3). The amount of CO_2 evolved after darkening in the presence of CA in the medium, as indicated by CO_2(D) in Figure 3D, agreed with the amount of C_i accumulated within the cells in the light determined by the silicone oil centrifugation method (Table I). Thus, CO_2(D)

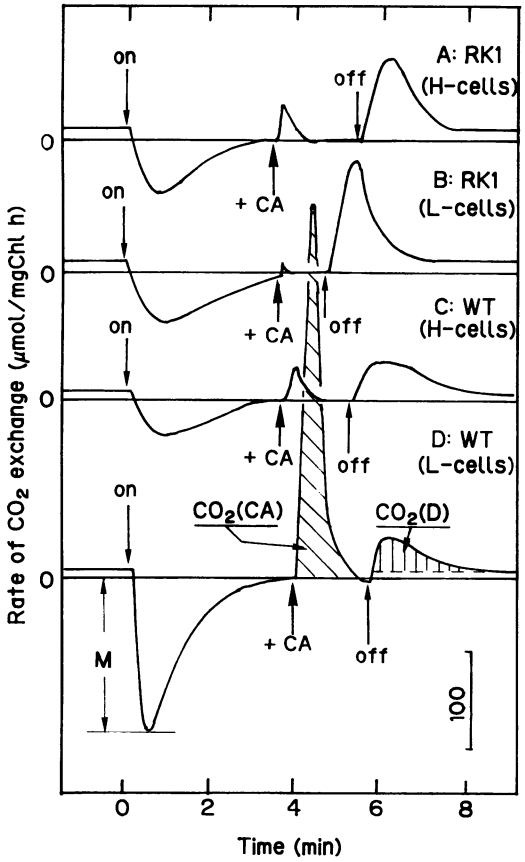

FIG. 3. Changes in CO_2 concentration in the gas phase of a cell suspension upon switching the white light (1100 μmol PAR/m^2·s) on and off and by addition of CA (40 μg/ml) in the light. A, H-cells of RK1; B, L-cells of RK1; C, H-cells of WT; D, L-cells of WT. The suspending medium as in Figure 2 except that IAc (3 mM) was added to the suspension of WT to inhibit the photosynthetic CO_2 fixation. IAc had no effect on the gas exchange profiles of RK1 and was not added to the suspension of the mutant. CA was added at the points indicated in the figure. M, CO_2(CA), and CO_2(D) on curve D indicate the maximal rate of CO_2 uptake, the amount of CO_2 released from the medium by addition of CA in the light, and the amount of CO_2 evolved after darkening, respectively.

Table I. *Amount of Intracellular C_i in L-Cells of WT Estimated from CO_2(D) and by Silicone Oil Centrifugation*

All the measurements were done at 21% O_2 in the presence of 3 mM IAc.

Experiments	Amount of Intracellular C_i	
	CO_2(D)	Silicone oil
	μmol/mg Chl	
1	2.2	2.1
2	1.5	1.7
3	1.8	1.3
4	1.5	1.8

reflects the size of the intracellular C_i pool before darkening. No significant difference was observed in CO_2(D) between H-cells and L-cells either for WT (Fig. 3, C and D) or RK1 (Fig. 3, A and B).

M, CO_2(CA) and CO_2(D) as a Function of the Period Exposed to Low CO_2. H-Cells of WT and RK1 were bubbled with air for various periods in the light and their CO_2 exchange patterns were obtained. M was low in H-cells of WT and started to increase after 4 h of exposure to low CO_2 to attain the highest value after 10 h (Fig. 4, curve C). During this period in low CO_2, M in WT increased up to 6.6 times. CO_2(CA) also increased during exposure of the cells to low CO_2, with the same time course as M (curve A). On the other hand, CO_2(D) remained nearly constant during 20 h of exposure of WT cells to low CO_2 (curve B). Thus, in WT of *Synechococcus*, the adaptation to low CO_2 did not affect the size of the intracellular C_i pool (measured in the absence of photosynthetic CO_2 fixation).

In contrast to WT, exposure of RK1 cells to low CO_2 did not have significant effect on their M and CO_2(CA) (curves A and C in Fig. 5). The M decreased slightly during 20 h of exposure to low CO_2 (curve C). The CO_2(CA) also decreased during this period; no CA-induced CO_2 evolution was observed with RK1 cells exposed to low CO_2 longer than 5 h (curve A).

The 42-kD Polypeptide in Cytoplasmic Membranes. Our previous studies have revealed that a 42-kD polypeptide was synthesized in the cytoplasmic membrane of *Synechococcus* during adaptation to low CO_2 (11, 12, 14). This was confirmed in this study, which shows a marked increase of the 42-kD polypeptide in L-cells of WT (Fig. 6, lanes a and b). The 42-kD polypeptide showed a weak band in the CBB-staining pattern of the cytoplasmic membrane from L-cells of RK1 (lane d). The density of the staining of this band was not much different from that of the

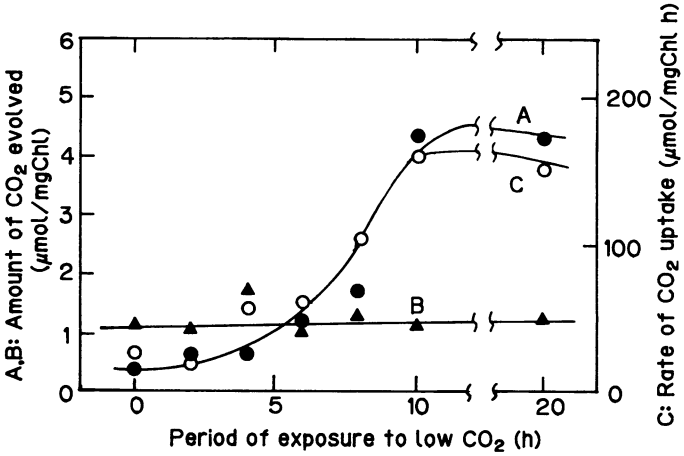

FIG. 4. M, CO_2(CA), and CO_2(D) in WT as a function of the period exposed to low CO_2 in the light (120 μmol PAR/m^2·s). A, CO_2(CA); B, CO_2(D); C, M. Gas exchange of the cells was measured in the presence of IAc under the conditions as in Figure 3.

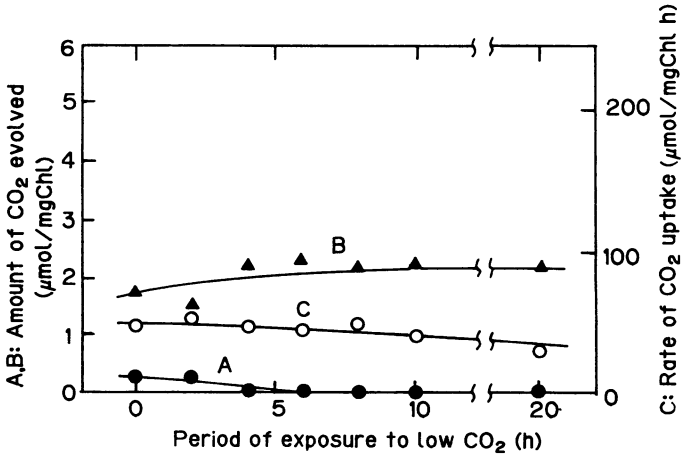

FIG. 5. M, CO_2(cA), and CO_2(D) in RK1 as a function of the period exposed to low CO_2 under the same light conditions as in Figure 4. A, CO_2(CA); B, CO_2(D); C, M.

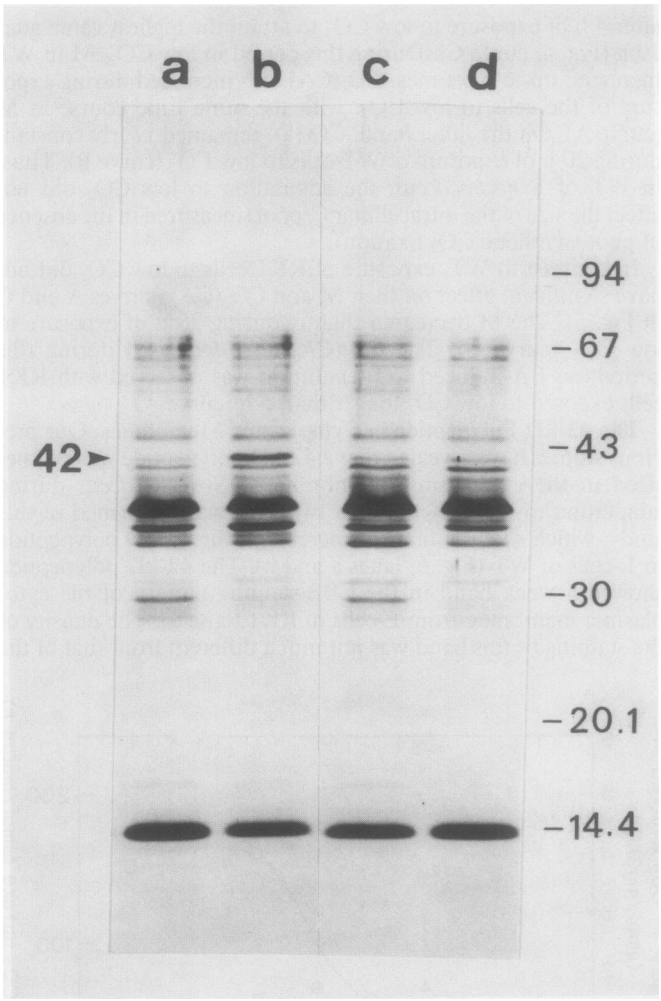

FIG. 6. Electrophoretic profiles showing CBB-staining patterns of polypeptides in the cytoplasmic membranes from H-cells (lanes a and c) and L-cells (b and d) of WT (a and b) and RK1 (c and d). Samples (15 μg protein each) were solubilized at room temperature for 30 min and were run in a 8 to 15% gradient SDS-polyacrylamide gel.

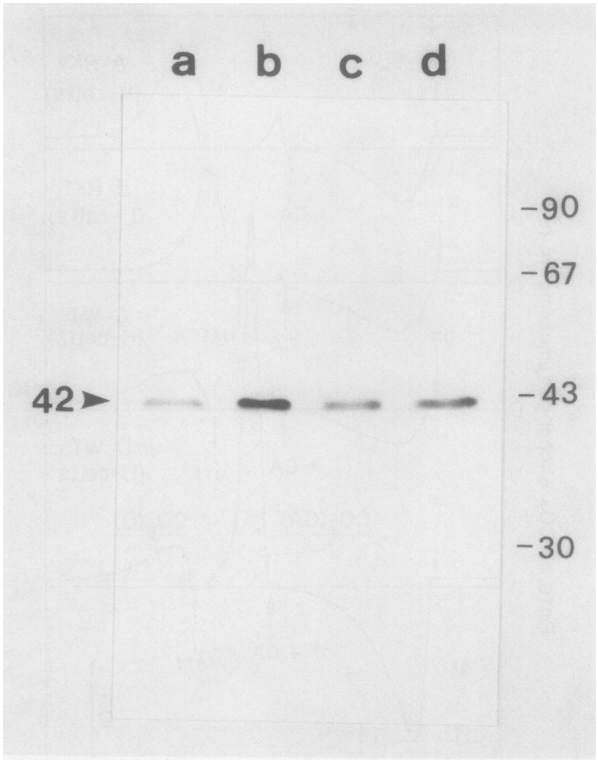

FIG. 7. Immunoblotting profiles of the polypeptides in the cytoplasmic membranes from H-cells (lanes a and c) and L-cells (b and d) of WT (a and b) and RK1 (c and d), obtained by using IgG against the 42-kD polypeptide. Samples (2.5 μg protein each) were solubilized at room temperature and were run in a 10% SDS-polyacrylamide gel. After electrophoresis the polypeptides in the gel were electrotransferred to nitrocellulose for immunostaining.

42-kD band in the pattern for H-cells of RK1 (lane c) or WT (lane a). This was more clearly demonstrated by the immunostaining of the same preparation, showing a band at 42 kD which reacted with the antibody against the 42-kD polypeptide (Fig. 7). The immunostaining of the 42-kD band in L-cells of RK1 was poor and was not much different from the staining of the band in H-cells of RK1 (lane c) or WT (lane a). In contrast, the 42-kD band for L-cells of WT (lane b) was strongly stained.

DISCUSSION

Exposure of H-cells of WT to low CO_2 caused following functional and compositional changes: (a) Decrease of the apparent $K_m(C_i)$ for photosynthesis (Fig. 2). (b) Increase of M and CO_2(CA) (Figs. 3 and 4). (c) Increase in content of the 42-kD polypeptide in the cytoplasmic membrane (Figs. 6 and 7). None of these changes occurred when H-cells of RK1 were exposed to low CO_2. Thus, RK1 is the mutant which is unable to adapt to low CO_2. The apparent $K_m(C_i)$ value for photosynthesis in H-cells of RK1 was 40 times higher than for H-cells of WT (Fig. 2), although the ability to take up C_i at air levels of CO_2 was similar for both types of cells (Fig. 3). The mutant E_1 of *Synechococcus* PCC7942 reported by Marcus *et al.* (5) also required high CO_2 for growth. In contrast to RK1, exposure of E_1 to low CO_2 resulted in the increases of M and in the amount of 42-kD polypeptide in the cytoplasmic membrane (14). Nevertheless E_1 mutant did not grow under low CO_2. These results indicate that the mutants RK1 and E_1 are defective in their ability to utilize the intracellular C_i pool for photosynthesis. Probably, these mutants do not have a component which enables the efficient production of CO_2 from the intracellular C_i pool at the site of carboxylation. Thus, RK1 appears to contain at least two mutations, one involves a component to transmit low CO_2 signal for inducing the functional and compositional changes observed in WT during low CO_2 adaptation and the other which involves a component which enables to utilize the intracellular C_i pool for photosynthesis. The E_1 mutant appears to contain the latter mutation but not the former one.

The growth rate of RK1 was as high as that of WT under high CO_2 (Fig. 1), although RK1 was unable to utilize the intracellular C_i pool for photosynthesis. Thus, photosynthesis in RK1 depends on extracellular supply of CO_2 by diffusion. This is also indicated by the high $K_m(C_i)$ value for photosynthesis in this mutant (Fig. 2). The concentration of CO_2 in the medium in equilibrium with 0.04% CO_2 in the gas phase is 11.9 μM at 30°C, which is far below the $K_m(CO_2)$ value for Rubisco of *Synechococcus* PCC7942, 300 to 400 μM (5). Evidently, the growth of WT at air levels of CO_2 is supported by CO_2 produced from the intracellular C_i pool. However, the fact that the growth rate of WT under these conditions is only one-fifth of the rate under high CO_2 (fig. 1) suggests that the rate of CO_2 production from the intracellular C_i pool is lower than that of CO_2 diffusion from the external medium under high CO_2.

The CA-induced CO_2 evolution clearly showed that a reaction driven by light produced the conditions in the medium where the concentration of HCO_3^- is higher than that expected under

the equilibrium conditions (Fig. 3D). In a previous paper, we inferred that the nonequilibrium is produced as a result of CO_2 influx and HCO_3^- efflux (9). It seems also possible that HCO_3^- is produced from CO_2 at the outer side of the cytoplasmic membrane and most of the HCO_3^- thus produced is released into the medium.

The amount of CO_2 evolved after darkening in the presence of CA in the medium [CO_2(D)] agreed with the size of the intracellular C_i pool (Table I). The result is in conflict with our previous data (8), which showed that the amount of CO_2 evolved after darkening in the absence of CA reflects the size of the intracellular C_i pool. Probably, the cells used in these previous experiments were not adapted to low CO_2 and CO_2(CA) was small in these cells. The incorrect conclusion in the previous paper led to an overestimation of the intracellular C_i pool (8, 11, 12), which must be corrected.

There was no significant difference between H-cells and L-cells of WT in the size of the intracellular C_i pool (Figs. 3 and 4). This suggests that the higher photosynthetic affinity for C_i in L-cells of WT as compared to that in H-cells (Fig. 2) is mainly due to higher ability of utilizing the intracellular C_i pool for photosynthesis. When CO_2(CA) is small, the influx of CO_2 from the gas phase into the cell suspension observed in the absence of CA in the medium is primarily due to active transport of C_i by the cells. Non-carrier-mediated CO_2 uptake may also occur as a result of the light-dependent alkalization of the intracellular space. The magnitude of the uptake dependent upon ΔpH is, however, negligibly small; the amount of C_i within the cells in equilibrium with 400 μl CO_2/L in the gas phase is only 0.027 μmol/mg Chl at pH 7.6, 30 C (the sorbitol impermeable space of 125 μl/mg Chl was assumed for WT in this calculation) (8).

The increases in M and CO_2(CA) observed in WT during low CO_2 adaptation (Figs. 3 and 4) can be explained in two ways: 1)Both the rates of C_i uptake and HCO_3^- efflux increased and 2) there was no change in the C_i-transporting activity and the activity of light-dependent conversion of CO_2 to HCO_3^- at the outer surface of the cytoplasmic membrane increased. It is, however, not known at present whether HCO_3^- is produced within the cells or at the cell surface.

The function of the 42-kD polypeptide is not yet understood. It is clear that the increase in M and CO_2(CA) during low CO_2 adaptation of WT (Fig. 4) is always accompanied with the increase in the amount of this polypeptide. Moreover, in RK1, neither CO_2(CA) nor the amount of the 42-kD polypeptide increased during exposure to low CO_2 (Figs. 3, 4, 6, and 7). These results suggest that the 42-kD polypeptide may function in taking up CO_2 and extruding HCO_3^- or producing HCO_3^- from CO_2 at the outer surface of the cytoplasmic membrane.

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