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Is There a Role for the 42 Kilodalton Polypeptide in Inorganic Carbon Uptake by Cyanobacteria?[1]

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ABSTRACT

Cyanobacterial cells accumulate substantial amounts of a membrane-associated 42 kilodalton polypeptide during adaptation to low CO_2 conditions. The role of this polypeptide in the process of adaptation and in particular in the large increase in the ability to accumulate inorganic carbon (C_i), which accompanies this process, is not yet understood. We have isolated a mutant *Synechococcus* PCC7942 that does not accumulate the 42 kilodalton polypeptide. The mutant requires a high-CO_2 concentration for growth and exhibits a very low apparent photosynthetic affinity for extracellular C_i. The latter might be attributable to the observed defective ability of the mutant to utilize the intracellular C_i pool for photosynthesis. The 42 kilodalton polypeptide does not appear to participate directly in the active transport of C_i, since the difference between the observed capabilities for CO_2 and HCO_3^- uptake of the mutant and the wild type is not sufficient to account for their different growth and photosynthetic performance. Furthermore, high CO_2-grown wild-type cells, where we could not detect the 42 kilodalton polypeptide, transported CO_2 faster than the mutant. An analysis of the curves relating the rate of accumulation of C_i to the concentration of CO_2 or HCO_3^- supplied, in the presence or absence of carbonic anhydrase, indicated that under the experimental conditions used here, CO_2 was the preferred C_i species taken up by *Synechococcus*.

A syndrome of structural, biochemical, and physiological changes is involved in the adaptation of cyanobacteria from a high (1–5% v/v CO_2 in air) to a low (air) level of CO_2 (1, 8–11). This syndrome leads to a large increase in the ability of the cells to actively accumulate C_i[2] and hence in the apparent photosynthetic affinity for extracellular C_i (1, 3–5, 9–15). The cells can therefore photosynthesize and grow at a high rate even though the $K_m(CO_2)$ of their Rubisco is some 20 times larger than the concentration of CO_2 at equilibrium with air (2). Very little information is as yet available on the molecular mechanism(s) of the adaptation to the various levels of CO_2 and of C_i uptake. An analysis of the polypeptide pattern in a membrane preparation as observed on SDS-PAGE has indicated a large accumulation of a 42 kD polypeptide in a cytoplasmic membrane-enriched fraction during the adaptation to low CO_2 (16, 17). The role, if any, of this polypeptide in C_i transport and accumulation is not yet understood, but it might be studied with the aid of a mutant that does not accumulate this protein. Should the protein indeed have a role in adaptation to low CO_2 or in C_i uptake and accumulation, such a mutant might not be able to adapt to or grow at the air level of CO_2. We have recently reported (10, 12, 17) on the physiological characters of a mutant of *Synechococcus* (E_1) that requires a high concentration of CO_2 for growth. The E_1 mutant is capable of accumulating the 42 kD polypeptide even when exposed to a CO_2 concentration as high as 0.3% (v/v in air) (17).

We have now isolated a new mutant of *Synechococcus* PCC7942 (*Anacystis nidulans* R_2), which apparently does not accumulate the 42 kD polypeptide, and have studied some of its physiological characters, as compared with those of the WT.

MATERIALS AND METHODS

The *Synechococcus* mutant O_{221} was isolated from a population of mutants that require high CO_2 for growth, using the same mutagenesis and enrichment protocols as previously described (12). Cells of the WT *Synechococcus* PCC7942 and the mutant O_{221} were grown at 30°C in a modified BG11 medium (21) containing 10 mM Hepes-NaOH, pH 7.0. The cultures were continuously illuminated by cool white fluorescent lamps at 5 $mW \cdot cm^{-2}$ and aerated with H, 5% CO_2 in air. L, low CO_2 conditions, were obtained by mixing air with CO_2-free air in 1:1 ratio. This treatment resulted in typical H and L adapted WT cells, respectively. Under the experimental conditions used here, an air level of CO_2 was too high to fully induce the low CO_2 syndrome, unlike the case of *Anabaena variabilis* (1, 5, 9) and other cyanobacteria (13). Since the mutant was unable to grow under L, and WT and the mutant were grown under H and transferred to L 15 h before the experiments.

The relation between the rate of photosynthesis and extracellular C_i concentration was determined with the aid of an O_2 electrode as previously described (9). Uptake of CO_2 and of HCO_3^- and their accumulation were measured by the filtering centrifugation technique (9, 11). Cells (50 μL) were suspended in 7.5 mM Bis-Tris-propane and 7.5 mM NaCl, corrected to pH 7.5 with Hepes and laid over a mixture of 1:1 bis(2-ethylexyl)phtalate and dibutyl phtalate (50 μL), instead of the silicon oil.

The apparent photosynthetic affinity of the mutant for extracellular C_i is very low compared to the WT (*cf.* Fig. 2). The amount of C_i present at the O_2 compensation point, in the case of the mutant, might therefore be relatively high. Decrease in the specific activity of the $^{14}C_i$ provided, due to dilution by the unlabeled C_i, might lead to a significant underestimation of the rate of C_i uptake in the case of the mutant. To overcome this problem, we kept the cell suspensions of the mutant and of L

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[2] Abbreviations: C_i, inorganic carbon; H, high CO_2 (5% v/v CO_2 in air); L, low CO_2 (a mixture of 1:1 air and CO_2-free air); Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; WT, wild type; CA, carbonic anhydrase.

WT at the O_2 compensation point, in two different O_2 electrode chambers. Aliquots (100 μL) of the mutant cell suspension were injected into the O_2 electrode chamber containing the culture of WT L. The amount of O_2 evolved by the cells gave a good indication of the amount of C_i present (in the mutant culture at the O_2 compensation point), assuming an O_2/CO_2 exchange ratio of 1:1. The amount of O_2 evolved following the injection of a known amount of C_i served to calibrate the system.

Isolation of the cytoplasmic membrane enriched fraction and the separation of the polypeptides on SDS-PAGE were carried out essentially as described by Omata and Ogawa (16). A sample of the crude homogenate, after the cells were broken in the French press, was used for the determination of the polypeptide pattern in the cell-free extract. The immumobloting was carried out essentially as described by Friedberg and Seijffers (6), following SDS-PAGE, using an antibody against the 42 kD polypeptide kindly provided by Omata and Ogawa (18). The activity of Rubisco as a function of the concentration of C_i was determined as previously described (12). Spheroplasts (25) isolated from the WT and the mutant were provided with 0.1 mM C_i in the light for 1 min and then were osmotically lysed into Rubisco assay medium (12) containing various concentrations of $^{14}C_i$. The reaction was terminated with acetic acid after 1 min.

RESULTS AND DISCUSSION

The rates of growth of the *Synechococcus* mutant O_{221} and the WT were similar when both were aerated with 5% CO_2 in air. Unlike the WT, however, the mutant required H for growth and was unable to grow in the presence of L or air (not shown, but see Ref. 12). A similar response has already been demonstrated for another mutant of *Synechococcus*, the E_1, where the requirement for an elevated CO_2 concentration for growth has been attributed to a defect in its ability to utilize the intracellular C_i pool for photosynthesis (12).

Absence of the 42 kD Polypeptide in a Cytoplasmic Membrane-Enriched Fraction of the O_{221} Mutant. We isolated a cytoplasmic membrane-enriched fraction from O_{221}, and WT cells exposed to H and L and compared the polypeptide patterns as observed following SDS-PAGE and Coomassie blue staining (Fig. 1a). A marked increase in the amount of a polypeptide with an apparent M_r of 42 kD was observed in the WT following the exposure to L (compare lanes A and B in Fig. 1a). In the mutant O_{221}, on the other hand (lanes C and D in Fig. 1a), we could not detect such an increase.

A Western blot of proteins in a cytoplasmic membrane-enriched fraction separated by SDS-PAGE (similar to that presented in Fig. 1a), as well as from cell free extract (lanes A to D and E to H, respectively), from H and L WT and O_{221} is presented in Figure 1b. The presence of the 42 kD polypeptide was observed in the case of the cytoplasmic enriched fraction (Fig. 1b, lane B) and of the crude extract from L WT (Fig. 1b, lane F). In H WT and the H and L mutants, on the other hand, we could hardly detect any polypeptide of apparent M_r of 42 kD. The Western blot of the cell-free extract in Figure 1b shows some reaction with heavier and lighter polypeptides. This could result from cross-reactivity between the antibody and other polypeptides as well as from the presence of a possible precursor, and of turnover products, in the cell extract. We are performing the required experiments to clarify these points, but the data in Figure 1 clearly indicate that the amount of the 42 kD polypeptide in the cytoplasmic enriched membrane and total protein preparations from O_{221} was much smaller. Thus, the O_{221} might be defective in the synthesis or the insertion of the 42 kD polypeptide to the cell envelope. The WT and another high CO_2 requiring mutant, the E_1, on the other hand, were able to accumulate the 42kD polypeptide (17). Therefore, the O_{221} and E_1 are clearly different types of high CO_2-requiring mutants.

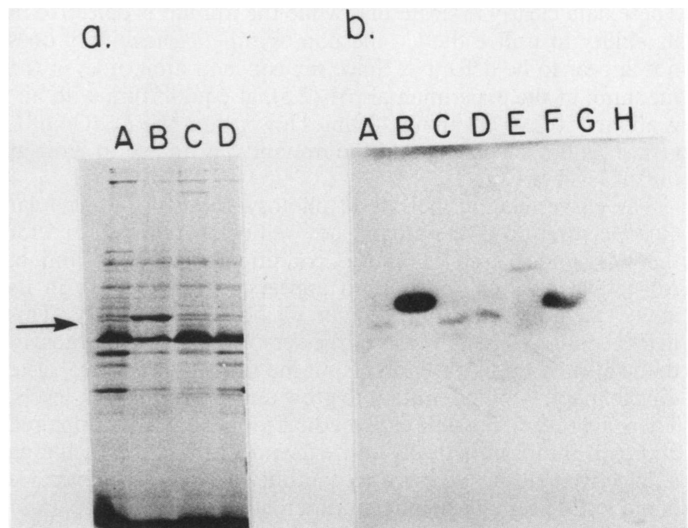

FIG. 1. (a) Coomassie blue staining and (b) immunoblot following the electrophoretic separation of the polypeptides in a cytoplasmic membrane-enriched fraction (lanes A–D) and in a crude extract of cell proteins (lanes E–H) from high-CO_2 maintained WT (A, E), L exposed WT (B, F), H maintained O_{221} (C, G), and L exposed O_{221} (D, H). *Synechococcus* PCC7942 WT and the mutant O_{221} were grown as described in "Materials and Methods" and aerated with 5% CO_2 in air. L exposed cells were aerated with a 1:1 mixture of air and CO_2-free air for 15 h. The gels were loaded with equal amounts of protein (100 μg) except for the immunoblot of the membrane fraction where 20 μg proteins were applied.

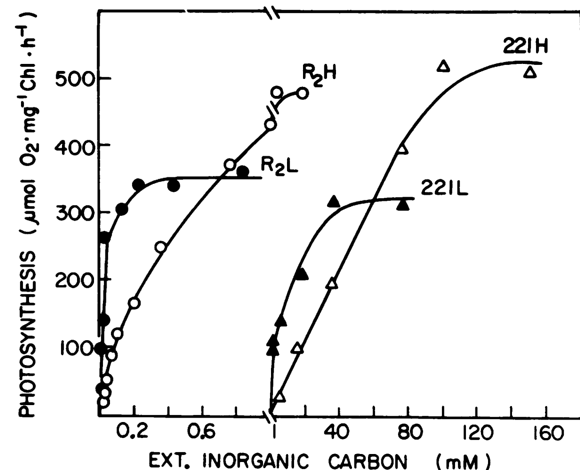

FIG. 2. The rate of photosynthetic O_2 evolution as a function of the extracellular C_i concentration in WT *Synechococcus* and the mutant O_{221}. The experiments were conducted at pH 8.0 in the O_2 electrode chamber, 30°C. Note the different scales for the C_i concentration in the case of the mutant.

Photosynthetic Response to the Extracellular and Intracellular Concentrations of C_i. The half-maximal photosynthetic rate ($K_{1/2}$) was reached by H O_{221} at a C_i concentration of 43 mM, *i.e.* about 100 times higher than that required by the H WT (Fig. 2). The WT and to some extent also the mutant undergo adaptation from high to low CO_2 as exhibited by the lower apparent photosynthetic $K_{1/2}$ (C_i) following the exposure of the cells to low CO_2 conditions for 15 h (Fig. 2). The $K_{1/2}$ (C_i) of the L O_{221}, however, was about 6 mM, while that of the L WT was only 15 μM.

The maximal photosynthetic rates (per Chl), at saturating C_i, were larger in H cells than in L cells, but under each set of conditions we could not detect any significant difference in the maximal photosynthetic rate as between the WT and the mutant.

These data clearly indicate that while the mutant is defective in its ability to utilize the C_i, the photosynthetic machinery does not appear to be defective. Since the concentration of C_i in the medium, at the experimental pH (7.5), at equilibrium with air, is about 150 μM, *i.e.* about 40 times lower than the $K_{1/2}$ (C_i) of L O_{221}, it is not surprising that the mutant was unable to grow at the air level of CO_2.

The curve relating the rate of photosynthesis to intracellular C_i concentration in the mutant lies well below that depicted for the WT under both H and L conditions (Fig. 3, a and b, respectively). Thus, the mutant appears to be defective in its ability to utilize the intracellular C_i photosynthetically. This defect could well be the basis of the very low apparent photosynthetic affinity for extracellular C_i in the mutant and hence, also, for the inability of the mutant to grow under limiting CO_2 levels. There are several possible explanations for the mutant's impaired ability to photosynthetically utilize the internal C_i pool, including a defective Rubisco with reduced affinity for CO_2. However, we could not detect any significant difference in the kinetic parameters of Rubisco, as between WT and the mutant, as determined following the lysis of spheroplasts to Rubisco assay medium (not shown, but see Ref. 12).

Uptake of Inorganic Carbon. Should the 42 kD polypeptide participate in the process of inorganic carbon transport, its absence in the mutant could explain the higher photosynthetic $K_{1/2}$ (C_i) and perhaps provide some information on the possible role of this polypeptide. The initial rates of CO_2 and HCO_3^- uptake as a function of their concentrations were determined in H and L WT and the mutant (Fig. 4). The curves for HCO_3^- uptake by H W.T and H O_{221} differ only slightly (Fig. 4a). When CO_2 was provided (Fig. 4c), the difference between H WT and H O_{221} was somewhat larger, but it was still too small to account for the very large difference in the apparent photosynthetic $K_{1/2}$ (C_i) in the WT and the mutant (Fig. 2). A similar conclusion may be drawn from the data obtained for L WT and mutant (Fig. 4b), although the initial rates of HCO_3^- and CO_2 uptake were larger than those obtained with H WT and mutant.

These experiments were conducted at pH 7.5, since at this pH the rate of uncatalyzed formation of CO_2 from HCO_3^- is very slow, and only about 0.8% of the supplied HCO_3^- will have been converted to CO_2 during the 5 s of the experiment (23). Furthermore, at this pH the concentration of the CO_2 species in the medium, at equilibrium, is only about 7% of the total C_i. The $^{14}C_i$ (HCO_3^- or CO_2) was provided after the cells had reached the O_2 compensation point. The concentration of C_i present in the medium at this point is considerably higher in the case of the mutant (300–400) μM, in different experiments, as compared with 10 to 30 μM for WT H and close to zero for WT L), most probably due to the higher photosynthetic $K_{1/2}$ (C_i) for the mutant (Fig. 2). The change in concentration and the specific activity of the $^{14}C_i$ provided has been corrected for by taking the concentration of the unlabeled C_i present into account. The presence of a relatively high external C_i concentration, at the O_2 compensation point, could also lead to inhibition of $^{14}C_i$ uptake due to competition between the different C_i species for transport. We did not study the interrelations between the different C_i species during transport in *Synechococcus*, but complex interaction and competition between the CO_2 and HCO_2^- have been reported in *Anabaena* (8, 24). Such interaction might be particularly pronounced in the case of $^{14}CO_2$ uptake by the mutant, since the concentration of HCO_3^-, the competing C_i species, is much higher than the CO_2 concentration. Part of the difference in C_i uptake exhibited by the WT and the mutant is therefore possibly attributable to the competition between the C_i species for transport in the latter.

Since the mutant accumulated C_i internally almost as effi-

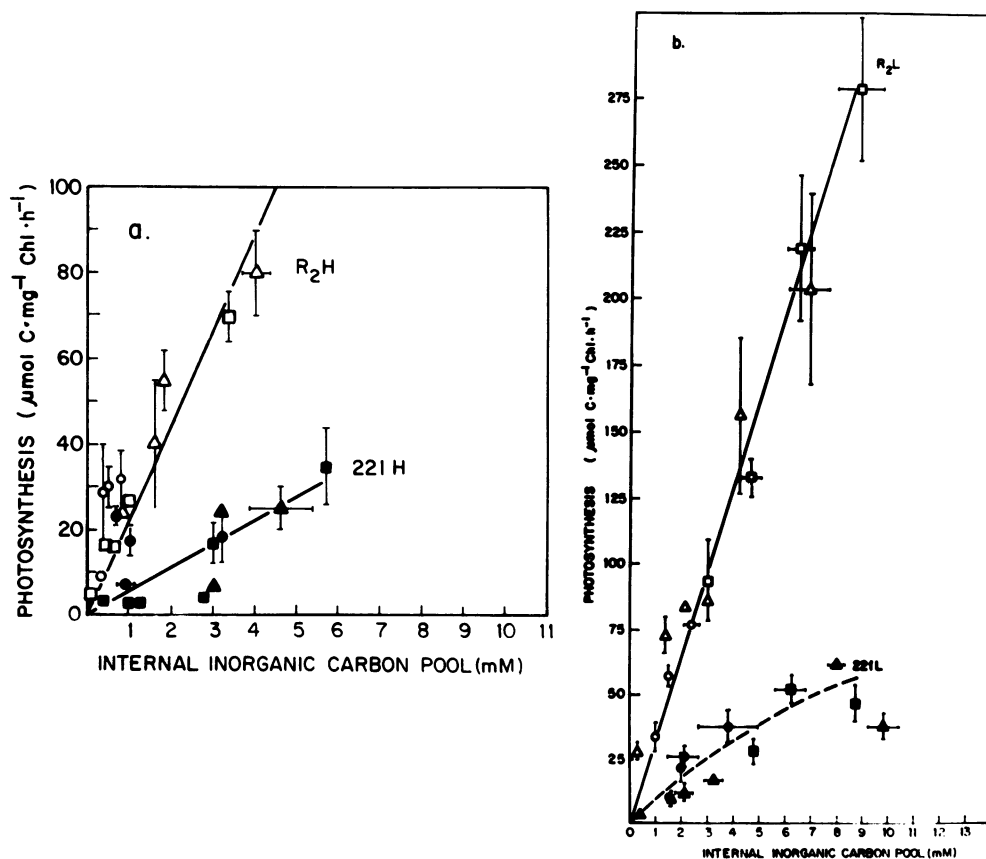

FIG. 3. The rate of acid stable ^{14}C accumulation (photosynthetic products) as a function of the intracellular $^{14}C_i$ concentration. (a) and (b) L exposed cells of the WT (open symbols) and of O_{221} (closed symbols) were provided with different concentrations of HCO_3^- (O, ●), HCO_3^- plus carbonic anhydrase (CA, □, ■), or CO_2 (△, ▲) for 5 s. Data (presented as the mean ± SD, $n = 5$) are from the same experiments where the uptake of CO_2 and HCO_3^- were determined (Fig. 4).

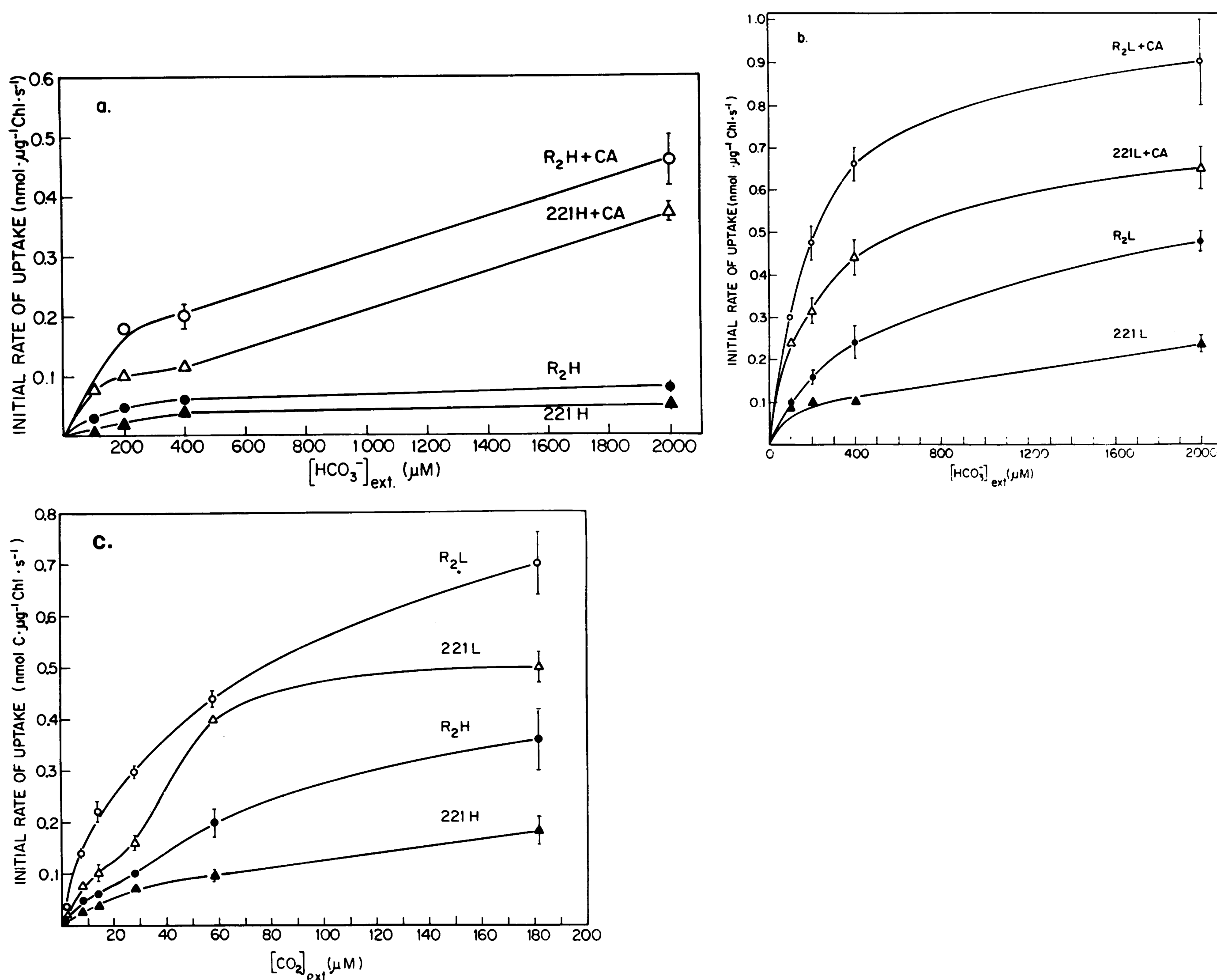

FIG. 4. Initial rate of C_i uptake as a function of the concentration of the C_i species supplied. (a) H and (b) L exposed cells of the WT (R_2) and the mutant O_{221} (221) were provided with HCO_3^- (a and b) in the presence or absence of CA or (c) with CO_2 for 5 s. See "Materials and Methods" for detailed experimental conditions.

ciently as did the WT (Figs. 3 and 4), we must conclude that the 42 kD polypeptide does not participate directly in C_i uptake. This conclusion is supported by the observation that though both H WT and H mutant lack the 42 kD polypeptide (Fig. 1b), the former exhibits superior ability to accumulate CO_2 (Fig. 4c). The 42 kD polypeptide might nevertheless have a significant role in some other process that is related to the low CO_2 syndrome, but this role remains to be established.

The nature of the defect in the O_{221} mutant is not yet understood. It is not known whether the membrane-associated 42 kD polypeptide, which was hardly detected in the mutant, is responsible for the defect in its ability to photosynthetically utilize the intracellular C_i pool. This, however, is not very likely since H WT, which also does not exhibit the 42 kD (Fig. 1), was able to utilize the intracellular C_i pool almost as efficiently as the L WT (compare Fig. 3, a and b).

Nature of the C_i Species Translocated by *Synechococcus*. The affinity of the C_i transport system for CO_2 was much higher than that for HCO_3^- in both the WT and the mutant (Fig. 4). Furthermore, a strong stimulation of HCO_3^- uptake was observed when CA was present in the medium presumably due to a stimulated rate of CO_2 formation. These findings could result from faster diffusion of the smaller and uncharged CO_2 molecule through the unstirred layer (cell envelope); alternatively, CO_2 might be the preferred C_i species for transport. Elucidation of the relative role of each of these factors might be deduced from the nature of the curves relating the rate of uptake to the concentration (Fig. 4). The maximal rate of C_i transport should not be affected by diffusion to the uptake site (19) but the data in Figure 4b clearly show that the V_{max} for HCO_3^- uptake was strongly affected by the presence of CA. It is therefore concluded that under the experimental conditions used here, CO_2 is likely to be the preferred C_i species taken up by *Synechococcus* (3, 4, 22).

It appears there may be a large species variation among cyanobacteria with regard to the relative roles of CO_2 and HCO_3^- in C_i uptake. The saturable nature of the rate *versus* concentration curves (Fig. 4) clearly indicates that both CO_2 and HCO_3^- are transported via a carrier. Compared with *Anabaena* (1, 8, 9, 24), however, HCO_3^- uptake by *Synechococcus* appears to be less significant for C_i uptake than for CO_2. A firm conclusion as to the relative roles of each C_i species would have to rest on

detailed analysis of the uptake parameters for each species and the competition between them during uptake, taking into account the relative abundance of each C_i species under the specific growth and experimental conditions.

The extended linear dependence of the photosynthetic rate on intracellular C_i (Fig. 3) suggests a low photosynthetic affinity for the dominant C_i species in the intracellular pool, *i.e.*, that the dominant species is HCO_3^-. Similar findings for *Anabaena* (1, 24) were interpreted as indicating that HCO_3^- was the C_i species arriving at the trans (inner) side of the membrane regardless of the C_i species provided. We have previously postulated a vectorial CA-like carrier as one of two systems concerned in C_i uptake in *Anabaena* (24) (see also Refs. 1 and 20), and the present evidence suggests the same model might also apply for *Synechococcus*. At present, it is not yet known whether, as suggested for *Anabaena*, two different systems for C_i uptake operate in *Synechococcus* (one for CO_2 only and the second transferring both CO_2 and HCO_3^-). The stimulation of C_i uptake when HCO_3^- was provided at saturating concentration, in the presence of CA (Fig. 4b) however, may suggest the operation of two distinct systems for HCO_3^- and CO_2. Some mode of activation by CO_2, resembling positive cooperativity would have to be postulated to explain the complex curve for CO_2 uptake, particularly the inflection point around 30 μM (Fig. 4c).

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