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Presence of the CO_2-concentrating mechanism in some species of the pyrenoid-less free-living algal genus *Chloromonas* (Volvocales, Chlorophyta)

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Abstract. Physiological and morphological characteristics related to the CO_2-concentrating mechanism (CCM) were examined in several species of the free-living, unicellular volvocalean genus *Chloromonas* (Chlorophyta), which differs morphologically from the genus *Chlamydomonas* only by lacking pyrenoids. The absence of pyrenoids in the chloroplasts of *Chloromonas* (*Cr.*) *rosae* UTEX 1337, *Cr. serbinowii* UTEX 492, *Cr. clatharata* UTEX 1970, *Cr. rosae* SAG 26.90, and *Cr. palmelloides* SAG 32.86 was confirmed by light and electron microscopy. In addition, immunogold electron microscopy demonstrated that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) molecules were distributed almost evenly throughout the chloroplasts in all five *Chloromonas* strains. However, *Chloromonas* exhibited two types of physiological characteristics related to the CCM depending on the species or strains examined. *Chloromonas rosae* UTEX 1337 and *Cr. serbinowii* had high photosynthetic affinities for CO_2 in cells grown in culture medium bubbled with air (low-CO_2 cells), compared with those grown in medium bubbled with 5% CO_2 (high-CO_2 cells), indicating the presence of the low-CO_2-inducible CCM. In addition, these two *Chloromonas* strains exhibited low-CO_2-inducible carbonic anhydrase (CA; EC 4.2.1.1) activity and seemed to have small intracellular inorganic carbon pools. Therefore, it appears that *Cr. rosae* UTEX 1337 and *Cr. serbinowii* possess the CCM as in pyrenoid-containing microalgae such as *Chlamydomonas reinhardtii*. By contrast, *Cr. clatharata*, *Cr. rosae* SAG 26.90 and *Cr. palmelloides* showed low photosynthetic affinities for CO_2 when grown under both CO_2 conditions. Moreover, these three strains exhibited an apparent absence of intracellular inorganic carbon pools and lacked low-CO_2-inducible CA activity. Thus, *Cr. clatharata*, *Cr. rosae* SAG 26.90 and *Cr. palmelloides*, like other pyrenoid-less algae (lichen photobionts) reported previously, seem to lack the CCM. The present study is the first demonstration of the CCM in pyrenoid-less algae, indicating that pyrenoids or accumulation of Rubisco in the chloroplasts are not always essential for the CCM in algae. Focusing on this type of CCM in pyrenoid-less algae, the physiological and evolutionary significance of pyrenoid absence is discussed.

Key words: Alga (green) – Carbonic anhydrase – CO_2-concentrating mechanism – Photosynthesis – Pyrenoid – Ribulose-1,5-bisphosphate carboxylase/oxygenase

Introduction

Many species of microalgae are capable of adapting to changes in the concentrations of inorganic carbon in their aquatic environment. When such algae are grown in the presence of 1–5% CO_2 (high-CO_2 cells) and then transferred to an air-level CO_2 concentration (low-CO_2 cells), the whole-cell affinity for CO_2 in photosynthesis increases markedly in order to maintain high photosynthetic activity (Aizawa and Miyachi 1986; Badger 1987). Such high affinity for CO_2 in low-CO_2 cells is attributable to the "CO_2-concentrating mechanism" (CCM), a process that concentrates inorganic carbon around the first CO_2-fixation enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), which exhibits low affinity for CO_2 (Badger 1987).

Carbonic anhydrase (CA; EC 4.2.1.1) and the intracellular inorganic carbon pool (Ci pool) have been intensively studied as factors related to the CCM (Aizawa and Miyachi 1986; Badger 1987; Tsuzuki and Miyachi 1989; Badger and Price 1994; Raven 1997). The CA activity in the periplasmic space enhances the uptake of CO_2 from the surrounding medium into microalgal

Abbreviations: CA = carbonic anhydrase; CCM = CO_2-concentrating mechanism; *Cd.* = *Chlamydomonas*; Ci pool = inorganic carbon pool; *Cr.* = *Chloromonas*; high-CO_2 cells = cells grown in the presence of 1–5% CO_2; low-CO_2 cells = cells grown in air-level CO_2; LS = large subunit

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cells (Tsuzuki and Miyachi 1989), and that located in the internal region of the cell is considered to supply CO_2 to Rubisco (Tsuzuki et al. 1980). Involvement of the Ci pool in the CCM has been demonstrated by both physiological studies and analyses of *Chlamydomonas reinhardtii* mutants (Badger et al. 1980; Spalding et al. 1983a, b; Badger 1987).

In addition to CA activity and the Ci pool, the pyrenoid has recently been regarded as one of the functional units of the CCM (Kuchitsu et al. 1991; Ramazanov et al. 1994; Smith and Griffiths 1996a,b). Pyrenoids are unique proteinaceous bodies generally surrounded by grains or sheaths of starch, located in the chloroplast stroma of most eukaryotic algae and of several species of hornworts (Anthocerotae) (Griffiths 1970; Okada 1992). Pyrenoids consist mainly of Rubisco (Holdsworth 1971; Salisbury and Floyd 1978; Satoh et al. 1984; Kuchitsu et al. 1991; Okada et al. 1991), and the Rubisco in chloroplasts is localized predominantly in the pyrenoids of pyrenoid-containing algae (Lacoste-Royal and Gibbs 1987; McKay and Gibbs 1989; Morita et al. 1997). Development of the pyrenoids in the low-CO_2 cells of *Chlorella*, *Scenedesmus* (Miyachi et al. 1986) and *Dunaliella* (Tsuzuki et al. 1986) indicates that these organelles are involved in the CCM (Kuchitsu et al. 1991; Ramazanov et al. 1994). In addition, recent comparative physiological studies of pyrenoid-containing and pyrenoid-less algae or bryophytes have indicated that the pyrenoids are related to the CCM (Palmqvist 1993; Palmqvist et al. 1994; Smith and Griffiths 1996a,b). However, differences in photosynthetic affinity for CO_2 between low- and high-CO_2 cells were not examined in these comparative studies. Moreover, the "pyrenoid-less" algal species thus examined were limited to photobionts (photosynthetic organisms present in the lichen).

The genus *Chloromonas* comprises free-living volvocalean algae, and differs morphologically from the genus *Chlamydomonas* only by the fact that its members lack pyrenoids (Ettl 1983). Thus, *Chloromonas* seems to be an ideal experimental alga lacking pyrenoids that is directly comparable with *Chlamydomonas* (*Cd.*) *reinhardtii*, one of the most intensively studied algae in relation to photosynthesis (see Harris 1989). However, to our knowledge, no photosynthetic studies of the genus *Chloromonas* have been carried out.

The present study was performed to characterize the CCM in several species or strains of the genus *Chloromonas* in comparison with *Cd. reinhardtii*. Photosynthetic affinity for CO_2, the intracellular Ci pool and CA activity were studied mainly on the basis of comparison between low-CO_2 and high-CO_2 cells. In addition, the absence of pyrenoids or lack of Rubisco accumulation in the chloroplasts of the *Chloromonas* strains were investigated by light microscopy, as well as transmission and immunogold electron microscopy.

Materials and methods

Algal cultures. *Chloromonas* (*Cr.*) *rosae* UTEX 1337, *Cr. serbinowii* UTEX 492 and *Cr. clatharata* UTEX 1970 were provided by the Culture Collection of Algae at the University of Texas at Austin (Starr and Zeikus 1993). *Chloromonas rosae* SAG 26.90 and *Cr. palmelloides* SAG 32.86 were obtained from Sammlung von Algenkulturen at the University of Göttingen (Schlösser 1994). *Chlamydomonas reinhardtii* 137c$^-$ was kindly provided by Dr. K. Shimogawara (Teikyo University, Japan). The cells were cultured in an oblong flat vessel containing 50 ml of 3/10 × HSM culture medium (Sueoka et al. 1967), bubbled with 5% CO_2 or ordinary air and continuously illuminated at 120 μmol photons m^{-2} s^{-1}. For culturing *Chloromonas* strains, 0.1 μg l^{-1} vitamin B_{12}, 0.1 μg l^{-1} biotin, and 10 μg l^{-1} thiamine HCl were added to the medium. Medium C (Ichimura 1971) was also used for culturing *Chloromonas* cells for electron microscopy. Preliminary experiments have shown that all the strains examined exhibited luxuriant growth at 23 °C. However, *Cr. clatharata*, *Cr. rosae* SAG 26.90 and *Cr. palmelloides* cultured at 23 °C did not swim actively and exhibited lower growth rates than at 15 °C. On the other hand, cells of *Cr. rosae* UTEX 1337 and *Cr. serbinowii* did not grow well at 15 °C. Therefore, cultures for the following physiological experiments were done at 15 °C and 23 °C for the former three strains of *Chloromonas*, and 23 °C for the latter two *Chloromonas* strains and *Cd. reinhardtii*. For electron microscopy, cells of all the strains were grown at 23 °C.

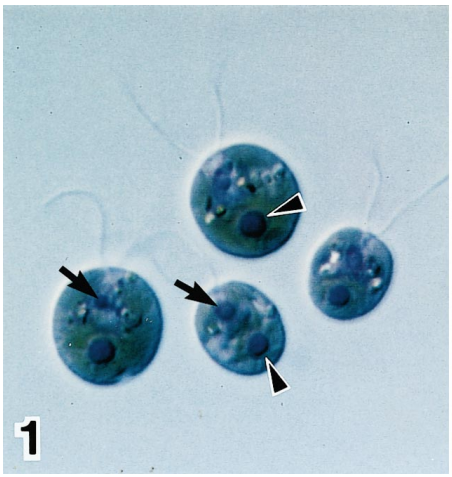
Light microscopy. Cells of *Chloromonas* strains and *Cd. reinhardtii* were stained with bromophenol blue in 0.1% $HgCl_2$ (see Kuchitsu et al. 1988) and observed using a BX 50 microscope equipped with Nomarski differential interference optics (Olympus Optical Co., Tokyo, Japan).

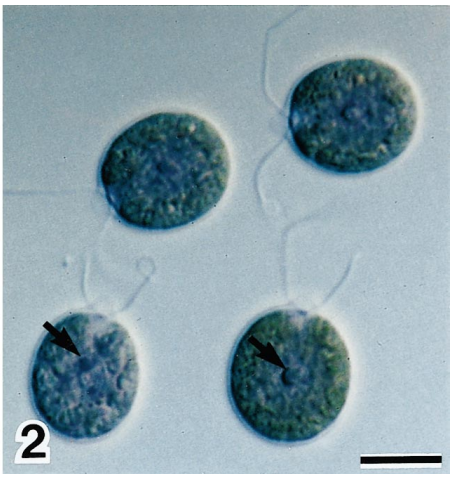
Electron microscopy and immunogold electron microscopy. Transmission electron microscopy and immunogold electron microscopy of Rubisco distribution were performed as described previously (Nozaki et al. 1994). The *Cd. reinhardtii* Rubisco large subunit (LS) antibody used for this immunogold electron microscopy cross-reacted specifically with the Rubisco LS (55 kDa) in all the *Chloromonas* strains in the preliminary immunoblot analyses.

Measurement of photosynthetic O_2 evolution. Photosynthetic O_2 evolution by cells was measured with a Clark-type oxygen electrode at a photosynthetic photon flux density of 900 μmol m^{-2} s^{-1}. Prior to measurement, the cells were collected by centrifugation and resuspended in the assay medium (20 mM 3-(N-morpholino)-propanesulfonic acid-NaOH, pH 8.0) to give a chlorophyll concentration of 3–6 μg ml^{-1}. Fifteen units of CA (bovine; Sigma Chemical Co., St. Louis, Mo., USA) were added to the cell suspension to maintain the dissociation equilibrium of dissolved inorganic carbon around the cells. Photosynthetic O_2 evolution was measured by injecting various concentrations of $NaHCO_3$ solution. $K_{0.5}(CO_2)$ values (external concentration of CO_2 at which the half-maximal rate of photosynthetic O_2 evolution is reached) were calculated from $NaHCO_3$-response curves, assuming that the pK_a for carbonic acid is 6.39 at 23 °C and 6.57 at 15 °C.

Measurement of CA activity. Cells were collected by centrifugation and resuspended in ice-cooled assay medium (20 mM sodium 5,5-diethylbarbiturate-H_2SO_4, pH 8.3), and then disrupted by sonication on ice. The CA activity of the resulting suspension or intact cells were measured according to the method of Yang et al. (1985). The chlorophyll content of the assay mixture was 10–20 μg ml^{-1} for *Cr. clatharata*, or 5–10 μg ml^{-1} for other strains.

Measurement of the intracellular inorganic carbon concentration. The intracellular inorganic carbon concentration, which represents the Ci pool, was determined using the silicone-oil-layer filtering centrifugation technique according to Tsuzuki (1986) with some modifications. The cells were suspended in the same medium as in the measurement of O_2 evolution to give a chlorophyll concentration of 20 μg ml^{-1}. The reaction was started by adding $^{14}NaHCO_3$ (2 GBq $mmol^{-1}$) solution to make a final concentration of 10 μM in the cell suspension, then incubated for 10, 20 or 40 s, and stopped by centrifugation for 10 s.





Figs. 1, 2. Light micrographs of *Chlamydomonas reinhardtii* (**Fig. 1**) and *Chloromonas rosae* SAG 26.90 (**Fig. 2**) cells stained with bromophenol blue in 0.1% $HgCl_2$. Note that no pyrenoids are evident in the chloroplasts of *Cr. rosae*. *Arrows* indicate nucleus; *arrowheads* indicate pyrenoid. Bar = 10 μm

Other methods. Protein was determined as described by Bradford (1976). Chlorophyll content was measured by the method of Wintermans and de Mots (1965).

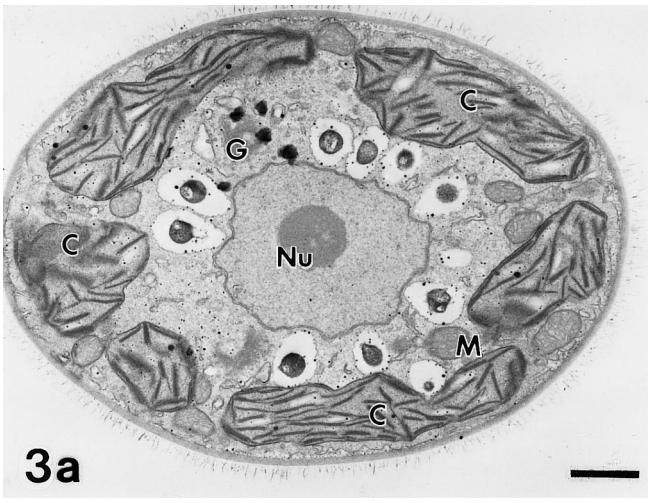
Results

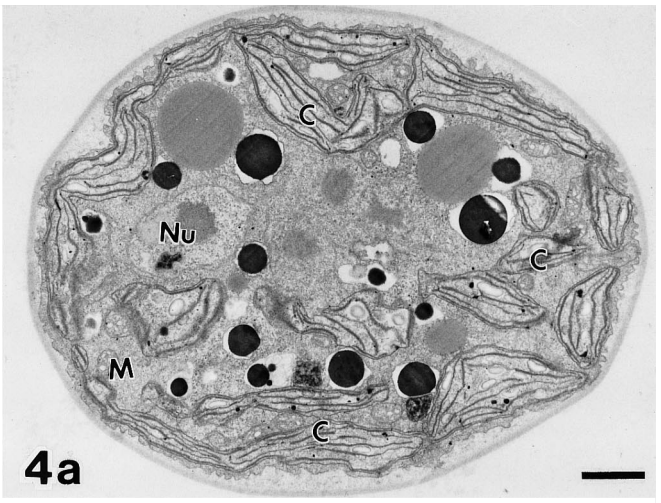
Light microscopy. Pyrenoids stained with bromophenol blue were found in the chloroplasts of the *Cd. reinhardtii* cells (Fig. 1). However, no pyrenoids were detected in any of the *Chloromonas* strains examined even when stained with bromophenol blue (Fig. 2).

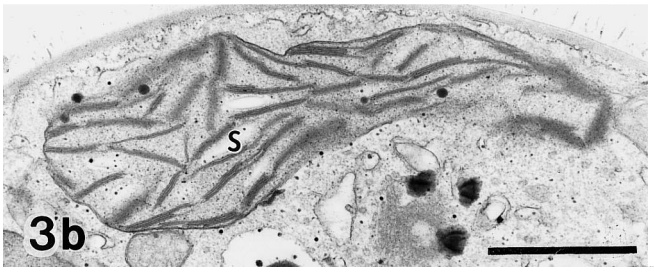
Figs. 3, 4. Transmission electron micrographs of cells of *Chloromonas rosae* UTEX 1337 (**Fig. 3a, b**) and *Cr. palmelloides* (**Fig. 4a, b**). **a** Longitudinal sections of cells. **b** Part of the chloroplast. *C*, chloroplast; *G*, Golgi body; *M*, mitochondrion; *Nu*, nucleus; *S*, starch. Bars = 1 μm

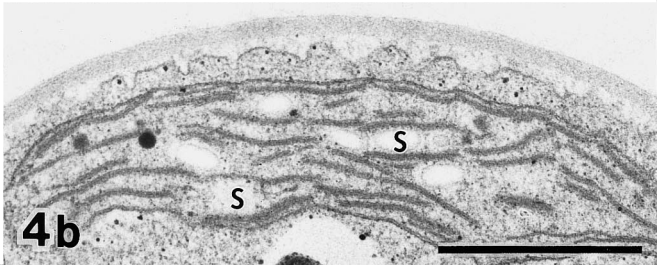
Electron microscopy and intracellular localization of Rubisco. In the sectioned cells of all *Chloromonas* strains, the nucleus was located in the center, and several to many chloroplast profiles were observed just beneath the cell membrane (Figs. 3–6). Although stroma starches were observed in the chloroplast, no traces of pyrenoid matrix or electron-dense bodies (see Nozaki et al. 1994) were recognized (Figs. 3, 4).

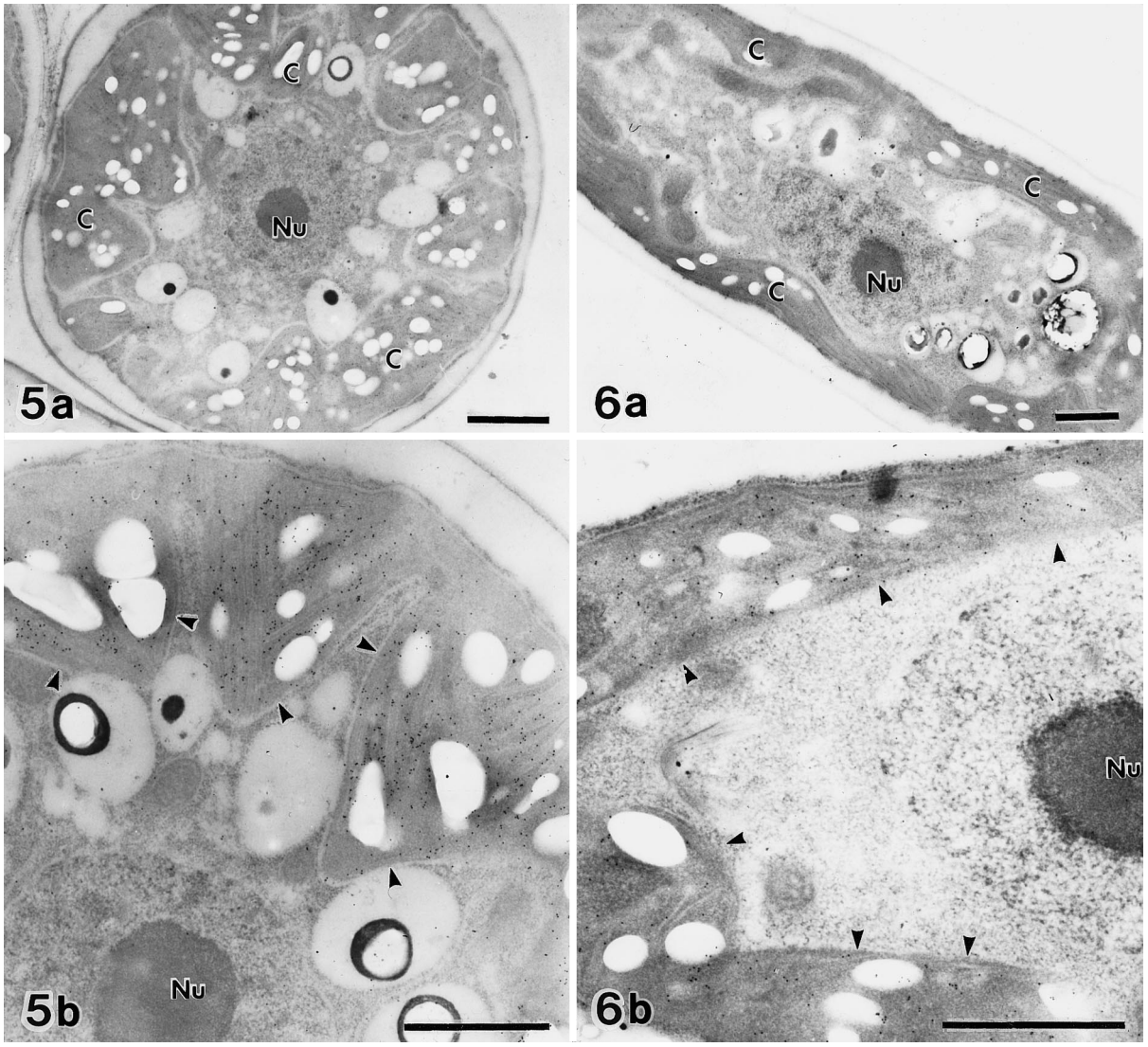
Immunogold electron microscopy demonstrated that the chloroplasts of all the *Chloromonas* strains were almost evenly labelled by anti-Rubisco LS and that none exhibited any accumulation of Rubisco (Figs. 5, 6). Table 1 shows the densities of gold particles for Rubisco LS over the various cell components. The densities of the gold particles in the chloroplast thylakoid region in all the *Chloromonas* strains were apparently higher than those in the cytoplasm. Such labelling was more or less











Figs. 5, 6. Immunogold localization of Rubisco in cells of *Chloromonas serbinowii* (**Fig. 5a, b**) and *Cr. clatharata* (**Fig. 6a, b**) using antibody raised against Rubisco LS from *Chlamydomonas reinhardtii.* Labelling by anti-Rubisco LS is recognizable in the chloroplasts. Note non-accumulation of Rubisco molecules in the chloroplast. **a** Longitudinal sections of cells. **b** Part of the chloroplast. *Arrowheads* indicate chloroplast membranes. *C*, chloroplast; *Nu*, nucleus. Bars = 1 μm

stronger than that in the thylakoid region of *Cd. reinhardtii* cells, but much weaker than in the pyrenoids of the *Cd. reinhardtii* (see Morita et al. 1997).

Photosynthetic affinity for CO_2. In *Cd. reinhardtii*, O_2 evolution at low $NaHCO_3$ concentration in the low-CO_2 cells was much higher than that in the high-CO_2 cells (Fig. 7a), whereas the maximal O_2 evolution in the low-CO_2 cells was slightly lower than that in the high-CO_2 cells (Table 2). Apparent $K_{0.5}(NaHCO_3)$ values for photosynthesis were 0.47 and 0.07 mM for the high- and low-CO_2 cells, respectively. These values correspond to 11.1 and 1.6 μM CO_2, respectively, at pH 8.0 (Table 2). The high- and low-CO_2 cells of *Cr. serbinowii* (Fig. 7b) and *Cr. rosae* UTEX 1337 showed essentially

Table 1. Densities of immunogold particles (μm^{-2} ± SD) of the anti-Rubisco LS in cell components of several species or strains of *Chloromonas* (*Cr.*), and *Chlamydomonas* (*Cd.*) *reinhardtii*

Species or strains	Pyrenoid	Thylakoid region	Cytoplasm	n^a
Cr. serbinowii UTEX 492	–	58.0 ± 17.2	2.7 ± 1.2	10
Cr. rosae UTEX 1337	–	49.3 ± 18.0	2.9 ± 1.4	10
Cr. clatharata UTEX 1970	–	49.5 ± 19.9	2.0 ± 1.0	10
Cr. rosae SAG 26.90	–	15.5 ± 3.1	0.9 ± 0.4	10
Cr. palmelloides SAG 32.86	–	12.1 ± 3.3	1.6 ± 0.9	10
Cd. reinhardtii 137c^{-b}	430.3 ± 78.2	9.0 ± 3.2	6.6 ± 2.3	20

[a]Number of cell sections analyzed
[b]Morita et al. 1997

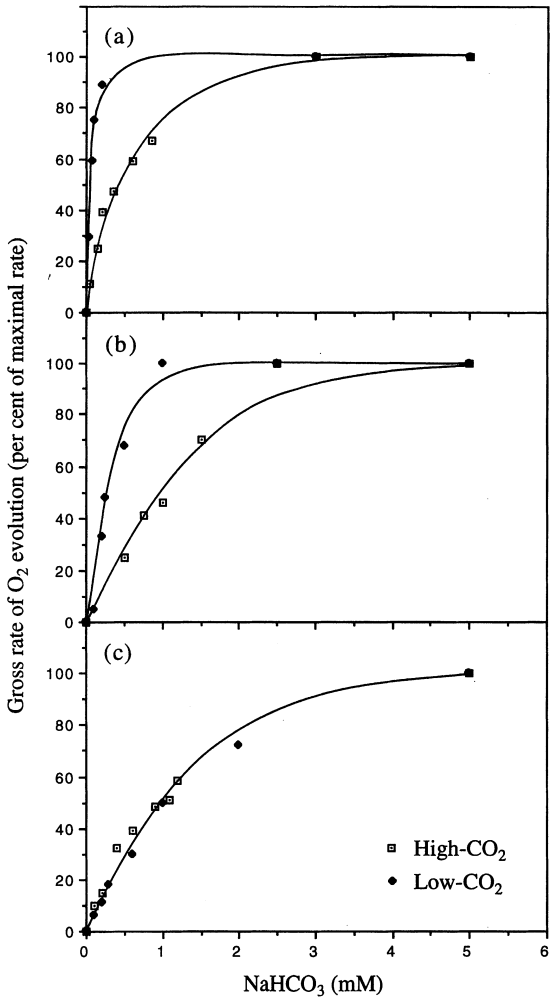


Fig. 7a–c. Response of photosynthetic O_2 evolution to $NaHCO_3$ concentration at pH 8.0 in cells grown under air (low-CO_2) or 5% CO_2 (high-CO_2). **a** *Chlamydomonas reinhardtii*; **b** *Chloromonas serbinowii*; **c** *Cr. clatharata*

the same $NaHCO_3$ dependency for photosynthesis as those of *Cd. reinhardtii* (Fig. 7a), although the $K_{0.5}(CO_2)$ values for photosynthesis in these strains were slightly higher than those of *Cd. reinhardtii*. The $K_{0.5}(CO_2)$ values for *Cr. serbinowii* and *Cr. rosae* UTEX 1337 were 18.6–19.5 and 4.0–6.2 μM for the high- and low-CO_2 cells, respectively (Table 2). In contrast, $NaHCO_3$ dependency in *Cr. rosae* SAG 26.90, *Cr. clatharata* (Fig. 7c) and *Cr. palmelloides* did not differ between the low- and high-CO_2 cells, and their $K_{0.5}(CO_2)$ values were constantly high (15.9–25.6 μM) (Table 2). In addition, these high $K_{0.5}(CO_2)$ values were not changed markedly by a shift of growth temperature from 15 °C (Table 2) to 23 °C (data not shown). Therefore, it would appear that high photosynthetic affinities for CO_2 were induced in the low-CO_2 cells of *Cr. rosae* UTEX 1337 and *Cr. serbinowii*, but not in those of *Cr. rosae* SAG 26.90, *Cr. clatharata* and *Cr. palmelloides*.

Carbonic anhydrase activity. In *Cd. reinhardtii*, *Cr. serbinowii* and *Cr. rosae* UTEX 1337, CA activities in the low-CO_2 cells were higher than those in the high-CO_2 cells, irrespective of whether the cells were intact or disrupted (Table 3). In these three strains, the CA activities of the intact cells (external CA activities) grown under low-CO_2 conditions were 5–15 times higher than those of the high-CO_2 cells, whereas the CA activities of disrupted cells (whole CA activities) grown under low-CO_2 conditions were 5–55 times higher than

Table 2. Maximal rates of photosynthetic O_2 evolution, $K_{0.5}(CO_2)$ value (external concentration of CO_2 at which the half-maximal rate of photosynthetic O_2 evolution is reached), and intracellular inorganic carbon concentrations after 10, 20 and 40 s of photosynthesis in several species or strains of *Chloromonas* (*Cr.*) in *Chlamydomonas* (*Cd.*) *reinhardtii*. $K_{0.5}(CO_2)$ value was determined from a $NaHCO_3$-response curve (see Fig. 7), assuming that pK_a for carbonic acid is 6.39 at 23 °C and 6.57 at 15 °C. Cells were grown under air (L) or 5% CO_2 (H) conditions. Data represent the mean values of triplicate experiments and SD (±) for maximal O_2 evolution and $K_{0.5}(CO_2)$, and the range of means of intracellular inorganic carbon concentration at each time point

Species or strains	CO_2 condition	Maximal O_2 evolution (μmol O_2 mg^{-1} Chl h^{-1})	$K_{0.5}(CO_2)$ (μM)	Intracellular inorganic carbon concentration (μM)
Cd. reinhardtii	L	105.0 ± 37.8	1.6 ± 0.4	0[a]–151
137c$^-$	H	112.1 ± 39.7	11.1 ± 2.2	–
Cr. serbinowii	L	137.0 ± 24.7	6.2 ± 0.6	0[a]–13
UTEX 492	H	156.0 ± 17.0	19.5 ± 0.6	–
Cr. rosae	L	147.8 ± 27.3	4.0 ± 0.2	10–27
UTEX 1337	H	210.9 ± 17.7	18.6 ± 0.8	–
Cr. clatharata	L	166.2 ± 26.0	23.6 ± 0.6	0[a]
UTEX 1970	H	234.3 ± 15.4	21.2 ± 0.5	–
Cr. rosae	L	148.4 ± 24.7	19.8 ± 0.5	0[a]
SAG 26.90	H	116.6 ± 31.5	21.6 ± 0.9	–
Cr. palmelloides	L	127.2 ± 10.6	18.2 ± 1.2	0[a]
SAG 32.86	H	173.3 ± 20.7	15.9 ± 1.1	–

[a]Not detected

Table 3. Effects of CO_2 concentration on CA activity during growth of several species or strains of *Chloromonas* (*Cr.*) and *Chlamydomonas* (*Cd.*) *reinhardtii*. Cells were grown under air (L) or 5% CO_2 (H) conditions. Data represent the mean values of triplicate experiments and SD (±)

Species or strains	CO_2 conditions	CA activity (units mg^{-1} Chl)	
		External	Whole
Cd. reinhardtii	L	12.2 ± 0.9	23.0 ± 3.1
137c$^-$	H	2.7 ± 0.8	5.0 ± 1.0
Cr. serbinowii	L	7.1 ± 0.6	10.1 ± 0.6
UTEX 492	H	< 0.5	2.0 ± 0.4
Cr. rosae	L	10.5 ± 5.0	59.6 ± 6.0
UTEX 1337	H	0.7 ± 0.04	1.1 ± 0.1
Cr. clatharata	L	2.8 ± 1.7	2.5 ± 1.1
UTEX 1970	H	1.9 ± 0.9	3.6 ± 2.1
Cr. rosae	L	6.0 ± 3.0	20.8 ± 1.9
SAG 26.90	H	5.5 ± 2.5	19.3 ± 2.7
Cr. palmelloides	L	4.8 ± 2.2	12.4 ± 3.2
SAG 32.86	H	9.1 ± 2.4	13.1 ± 4.3

those of high-CO_2 cells. In contrast, *Cr. rosae* SAG 26.90, *Cr. clatharata* and *Cr. palmelloides* showed no change in either external or whole CA activities, irrespective of CO_2 conditions during growth at both 15 °C (Table 3) and 23 °C. In these strains, the CA activities were much lower in cells grown at 23 °C (< 3.0 units mg^{-1} chl.) than in those grown at 15 °C (Table 3).

The Ci pool. Intracellular inorganic carbon was detected during photosynthesis in cells of *Cr. rosae* UTEX 1337 and *Cr. serbinowii*, although the concentrations were lower than those in *Cd. reinhardtii cells* (Table 2). Therefore, these two strains of *Chloromonas* seem to possess small intracellular Ci pools. In contrast, intracellular inorganic carbon was not detected during photosynthesis in cells of *Cr. rosae* SAG 26.90, *Cr. clatharata* and *Cr. palmelloides* (Table 2), indicating Ci pools were totally absent in these three strains.

Discussion

The present study using light microscopy, as well as transmission and immunogold electron microscopy, demonstrated that all five strains of *Chloromonas* lack pyrenoids and show no accumulation of Rubisco in their chloroplasts (Figs. 2–6). However, these algae exhibited two types of CO_2-use efficiency depending upon the species or strains examined. *Chloromonas rosae* SAG 26.90, *Cr. clatharata* and *Cr. palmelloides* demonstrated low photosynthetic affinities for CO_2 under low- and high-CO_2 conditions and complete absence of intracellular Ci pools (Table 2). In addition, CA activities in these three strains were unaffected by CO_2 conditions during growth (Table 3). Therefore, *Cr. rosae* SAG 26.90, *Cr. clatharata* and *Cr. palmelloides* apparently lack the CCM. This situation is essentially consistent with that of the pyrenoid-less photobionts and bryophytes examined in previous studies, whose correlative absence of pyrenoids and the CCM was demonstrated (Palmqvist 1993; Palmqvist et al. 1994; Smith and Griffiths 1996a, b). In contrast, the remaining two strains, *Cr. rosae* UTEX 1337 and *Cr. serbinowii*, exhibited low-CO_2-inducible high photosynthetic affinities for CO_2, correlated with the increase in CA activity (Tables 2, 3). In addition, these two strains may possess small intracellular Ci pools (Table 2). Therefore, *Cr. rosae* UTEX 1337 and *Cr. serbinowii* apparently possess the CCM, even though they lack pyrenoids. Since pyrenoids are suggested to play a significant role in the CCM in pyrenoid-containing algae (Kuchitsu et al. 1991; Palmqvist et al. 1994; Ramazanov et al. 1994; Smith and Griffiths 1996a,b), the CCM in *Cr. rosae* UTEX 1337 and *Cr. serbinowii* may differ from that of pyrenoid-containing algae in that the pyrenoids are not involved. The present study is the first to demonstrate the presence of the CCM in pyrenoid-less algae, clearly indicating that pyrenoids are not always essential for the CCM in algae.

Since intracellular Ci pools have not previously been recognized in the pyrenoid-less photobionts and bryophytes, it is suggested that pyrenoids are involved in formation of the Ci pools that represent the CCM (Palmqvist et al. 1994; Smith and Griffiths 1996a,b). However, the two CCM-containing strains, *Cr. rosae* UTEX 1337 and *Cr. serbinowii* seem to possess pyrenoid-unrelated, small Ci pools. Recently a new model of the CCM was proposed to explain the ability in some species of algae such as the marine diatom *Phaeodactylum tricornutum* to concentrate CO_2 around the Rubisco without a large accumulation of intercellular inorganic carbon (Raven 1997). The CCM in these two *Chloromonas* strains may be considered based on this model.

Induction of CA activity under low-CO_2 condition and its contribution to the CCM have been reported in many pyrenoid-containing algae (see Badger and Price 1994). On the other hand, *Coccomyxa* possesses significantly higher internal CA but lacks the CCM, and CA inhibitor had no effects on the photosynthetic affinity for CO_2 in this alga, indicating that CA activity in *Coccomyxa* is unrelated to the CCM (Palmqvist et al. 1995). In addition, it is suggested that low-CO_2-inducible mitochondrial CA in *Cd. reinhardtii* cells (Eriksson et al. 1996) is not involved in photosynthesis, indicating that CA and CA functions are diverse (see Hiltonen et al. 1995; Karlsson et al. 1995; Hewett-Emmett and Tashian 1996). Thus, CA activity induced under low-CO_2 condition does not always correlate with photosynthesis or a CCM. However, in the present study, two *Chloromonas* strains (*Cr. rosae* UTEX 1337 and *Cr. serbinowii*) with the CCM exhibited induction of CA activity under low-CO_2 conditions, whereas three other *Chloromonas* strains lacking the CCM did not exhibit such induction of CA activity. Therefore, it is considered that the low-CO_2-inducible CA activity in *Chloromonas* contributes to the CCM.

Chloromonas rosae SAG 26.90, *Cr. clatharata* and *Cr. palmelloides* are similar to the lichen photobionts such as *Coccomyxa* in that they lack both pyrenoids and the CCM. These three strains of *Chloromonas* were

originally isolated from the frigid zone or snow (Starr and Zeikus 1993; Schlösser 1994), and photobionts originate from lichens. Therefore, it is speculated that these algae lacking both pyrenoids and the CCM may be slow-growing organisms adapted to those environments in which the CCM might not be necessary for survival (see Honegger 1991). On the other hand, biochemical analysis indicates that *Coccomyxa* has Rubisco that is more efficient than that of *Cd. reinhardtii* (Palmqvist et al. 1995), thus, it is also speculated that the absence of both pyrenoids and the CCM in the three *Chloromonas* strains, as well as *Coccomyxa*, may be based on such an increased biochemical efficiency of Rubisco molecules. Studies on Rubisco characteristics in *Chloromonas* are needed. The pyrenoid-less algae possessing the CCM (*Cr. rosae* UTEX 1337 and *Cr. serbinowii*) investigated in the present study originate from the temperate zone (Starr and Zeikus 1993), and therefore might have developed some unknown functions related to the CCM during evolution, in order to compensate for the lack of accumulation of Rubisco or pyrenoids in their chloroplasts.

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