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Photosynthesis and low CO_2 inducible protein synthesis in a newly isolated high CO_2-preferring mutant of *Chlamydomonas reinhardtii**

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SUMMARY: The effect of external CO_2 concentrations on the protein synthesis in *Chlamydomonas reinhardtii* wild-type is compared with that of a new high CO_2-preferring mutant, designated as *pyr*-45. Radiolabeled wild-type and *pyr*-45 cells exhibit up-regulation of two polypeptides (42-45 kDa) when adapted from high (5% CO_2 in air) to low CO_2 (0.03%), and wild-type induces three new ones (21, 36 37 kDa), but *pyr*-45 induces no new polypeptides. Total proteins from *pyr*-45 mutant cells do not crossreact with antibodies against the three low CO_2-inducible polypeptides of wild-type. The CO_2 requirement for half maximal rates of photosynthesis decreases when *pyr*-45 cells are switched from high to low CO_2, but not to the extent of wild-type cells. When exogenous carbonic anhydrase (CA) is added to these partially adapted cells, the CO_2 requirement is further reduced, but still not completely. The up-regulation of the 42-45 kDa polypeptides under low CO_2 growth conditions suggests these changes play a role in the adaptation of algal cells to limiting CO_2 concentrations in the environment and in the function of the CO_2 concentrating mechanism (CCM) in *Chlamydomonas reinhardtii*.

Key words: Adaptation, carbonic anhydrase, *Chlamydomonas*, CO_2-concentrating mechanism, mutant, photosynthesis, protein synthesis.

INTRODUCTION

The unicellular green alga *Chlamydomonas reinhardtii*, like many other algae, induces the CO_2-concentrating mechanism (CCM) when grown on limiting CO_2 concentrations (Badger *et al.*, 1980; Spalding *et al.*, 1983a,b,c; Moroney and Mason, 1991; Coleman, 1992). The nature of the CCM is not completely understood and several models have been proposed (Coleman, 1992; Ramazanov and Cárdenas, 1992). The clarification of the specific role played by carbonic anhydrase (CA) in regulation of the inorganic carbon nutrition and the CCM in cells is complicated by the fact that several isoforms of CA have been found in algae (Pronina *et al.*, 1981; Husic and Marcus, 1994; Fukuzawa *et al.*, 1990; Rawat and Moroney, 1991; Sultemeyer *et al.*, 1993). These isoforms of CA differ not only in intracellular location but also in their activity, which is dependent to varying degrees on the culture conditions, in particular CO_2 concentration (Pronina *et al.*, 1981; Fukuzawa *et al.*, 1990). At least 5 other polypeptides that are either absent or present in low

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amounts in cells grown on high CO_2 concentrations and induced during adaptation of *C. reinhardtii* cells to low CO_2 conditions (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Spalding *et al.*, 1991). These proteins include two polypeptides of approximately 42-45 kDa, two membrane-associated proteins with molecular weights of 36 kDa (LIP-36) and 21 kDa (LIP-21) (Husic and Marcus, 1994; Manuel and Moroney, 1988; Spalding *et al.*, 1991; Geraghty *et al.*, 1990; Spalding and Jeffrey, 1989; Ramazanov *et al.*, 1993), and 37 kDa-soluble polypeptide, which has been identified as a subunit of periplasmic CA (Coleman *et al.*, 1984a,b; Fukuzawa *et al.*, 1990). LIP-36 has been shown to be specifically localized in the chloroplast envelope membranes isolated from low CO_2-grown *C. reinhardtii* cells (Ramazanov *et al.*, 1993). However, none of the low CO_2-induced polypeptides have been linked to a specific function in the CCM (Spalding *et al.*, 1991).

A number of *C. reinhardtii* mutants that lacks low CO_2-inducible proteins have been isolated and shown not to grow under low CO_2 conditions (Spalding *et al.*, 1983a,b,c, 1991; Moroney *et al.*, 1989). Among these mutants, *cia*-5, synthesizes none of the low CO_2-inducible proteins (Moroney *et al.*, 1989) nor low CO_2-inducible mRNA's (Spalding *et al.*, 1991) when placed in low CO_2 conditions. Manuel and Moroney (1988) reported that the high CO_2-requiring mutant strain, designated as *pmp*-1 (Spalding *et al.*, 1983b), lacks two low CO_2 inducible polypeptides of approximately 42-45 kDa. In addition, Spalding *et al.*, (1991) provided evidence that the induction of these 42-45 kDa polypeptides in the wild-type represents an up-regulation in the synthesis of the polypeptides rather than *de novo* induction of new polypeptides. According to Spalding *et al.*, (1991) the *pmp*-1 lacks the up-regulation, although the 42-45 kDa polypeptides are present in this strain (Spalding *et al.*, 1991). Isolation of the mutant strain that is defective in these 42-45 kDa and/or induces only these two polypedtides could add a significance meaning to our understanding of the actual role of these proteins (if any) in the CCM.

In this work the effect of CO_2 concentration on the protein synthesis of wild type *C. reinhardtii* and in a newly isolated high CO_2-preferring mutant strain *pyr*-45 is studied. Labelling wild-type cells with $^{35}SO_4^{-2}$ shows the induction of 21, 36, 37, and 42-45 kDa polypeptides, while mutant cells induced only two polypeptides of 42-45 kDa. We suggest that the up-regulation in the synthesis of the 42-45 kDa polypeptides under limiting CO_2 conditions might play a role in the adaptation to limiting CO_2 concentrations in the environment and in the function of the CCM in *C. reinhardtii*.

MATERIAL AND METHODS

Algal strains and culture conditions

The wild-type strain of *Chlamydomonas reinhardtii* 6145c is a gift from Prof. Emilio Fernández (University of Córdoba, Spain), and high CO_2-preferring mutant *pyr*-45 cells have been isolated via UV mutagenesis. Algae were grown in minimal medium (Sueoka, 1960) in a specially constructed glass bioreactor with plane-parallel walls (0.5 cm inside) illuminated with 400 µmol $m^{-2}s^{-1}$ and aerated with either a high CO_2:air mixture (5:95, v/v) or with low CO_2 (air containing 0.03% $C0_2$).

Mutant isolation

High CO_2-preferring mutants were isolated following UV mutagenesis. High CO_2-grown wild-type cells were exposed to UV light for different times and aliquots of the cell suspension were plated onto minimal medium. After 24 h dark exposure these plates were illuminated (200 µmol $m^{-2}s^{-1}$) for two weeks in a high CO_2 chamber. After two weeks colonies were picked and transferred to new plates with minimal medium and exposed to the same light intensity in the growth chamber aerated with 0.03% CO_2. Colonies that grew poorly in low CO_2 were picked up and used for further analysis (see also results).

Labelling cells with $^{35}SO_4^{-2}$

Protein labelling with $^{35}SO_4^{-2}$ was performed according to Manuel and Moroney (1988). Cells previously grown on minimal medium were centrifuged and resuspended in minimal medium with 1/10 $MgSO_4$ concentration, aerated with 5% CO_2. After two days of cultivation cells were harvested by centrifugation at 5000 g for 5 min. The pellet was washed twice with growth medium lacking sulfate and resuspended in growth medium without sulfate to a chlorophyll concentration of 3-4 µg ml^{-1}. Cells were bubbled with low or high CO_2 and 15 µCi of carrier-free $H_2{}^{35}SO_4$ (1000 Ci/mmol) was added to

the cultures. After incubation for 6 h with $^{35}SO_4^{-2}$ cells were harvested by centrifugation at 5000 g for 5 min and the pellet was washed 3 times with 30 ml of 30 mM Hepes-KOH, pH 7.5 and resuspended in the buffer. To compare different treatments, samples were loaded to equal counts (250,000 count min^{-1} per lane). Autoradiography was performed using Kodak X-OMAT film. The amount of radioactivity incorporated into the algal cells was determined by taking aliquots of cells in buffer and counting the sample using a Beckman LS 1801 liquid scintillation counter.

Photosynthesis assays

Photosynthesis of algal cells was measured in 1-ml samples with an oxygen electrode (Hansatech Ltd., Norfolk, England). Algae were centrifuged at 5,000 g for 5 min, resuspended in 1 ml of 25 mM Hepes-KOH (pH 7.3) to the Chl concentration of 10 µg, and transferred to the electrode chamber, where they were allowed to consume the dissolved inorganic carbon (DIC) of the buffer and intracellular pool until no net photosynthesis was observed. Bicarbonate was added when net oxygen evolution had levelled off.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed with 12% (w/v) acrylamide concentration and/or gradient gel from 10 to 20% acrylamide concentration (Laemmli, 1970). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories except that 5% non-fat dry milk was used to block the nitrocellulose. Goat anti-rabbit IgG(H+L) horseradish peroxidase conjugate and HRP color development reagent were purchased from Bio-Rad Laboratories.

Polyclonal antibodies raised against a 37 kDa periplasmic CA of *C. reinhardtii* were kindly provided by Dr. James V. Moroney (Louisiana State University, USA) and antibodies raised against low CO_2 inducible LIP-21 polypeptide were a gift from Prof. Martin Spalding (Iowa State University, USA). Protein concentration was estimated according to Bradford (1976). Chlorophylls were extracted with absolute ethanol and quantitated using the absorption coefficients given by Wintermans and de Mots (1965).

TABLE 1. – Half-maximal photosynthetic rate $K_{0.5}(CO_2)$ values of *C. reinhardtii* wild-type and *pyr*-45 cells grown under different CO_2 concentrations. Photosynthesis measured in 25 mM Hepes-KOH, pH 7.3 and light intensity 400 µmol $m^{-2}s^{-1}$.

Growth conditions	Wild type	*pyr*-45
	$K_{0.5}(CO_2)$ µM	
5% CO_2	40±5	40±5
0.03% CO_2	2±1	10±2
1 µg CA	2±1	6±1

RESULTS

Photosynthesis assay

When *C. reinhardtii* cells grown on high concentrations of CO_2 were switched to low CO_2 conditions the algal cells required 5-6 h to adapt to the limiting CO_2 conditions. During this transition the apparent affinity of the cells for CO_2 increased (Table 1). The concentration of CO_2 required for half maximal rates of photosynthesis [$K_{0.5}(CO_2)$] in high CO_2-grown cells is about 40 µM CO_2 in both strains, while the low CO_2-grown wild-type cells required about 2±1 µM and mutant cells about 10±1 µM CO_2 (Table 1). The high affinity for DIC shown by *C. reinhardtii* wild-type cells grown in low CO_2 clearly indicates that these cells induce the CCM. *Pyr*-45 also was able to adapt partly to limiting CO_2 concentrations in the environment. The addition of 1 µg of bovine CA to the cells decreased the [$K_{0.5}(CO_2)$] for photosynthesis from 10 to 6±1 µM of CO_2 in the low CO_2-grown *pyr*-45 cells but not in wild-type irrespective of the growth conditions, nor in high CO_2-grown *pyr*-45 cells.

Labelling with $^{35}SO_4^{-2}$

Figure 1 shows an autoradiogram of newly synthesized proteins from cells grown either with low or high CO_2, enabling identification of proteins that are preferentially synthesized under low CO_2 conditions. $^{35}SO_4^{-2}$ labelling shows that at least 5 polypeptides with molecular weights of approximately 21, 36, 37 and 42-45 kDa were induced by low CO_2 in wild-type, while mutant cells induced only two polypeptides of 42-45 kDa. A small amount of 42-45 kDa polypeptides were also present in high CO_2-grown cells. Therefore, the increased amount of these polypeptides in both the wild-type and mutant cells represent an up-regulation in the synthesis of the polypeptides rather than a *de novo* induction of new polypeptides.

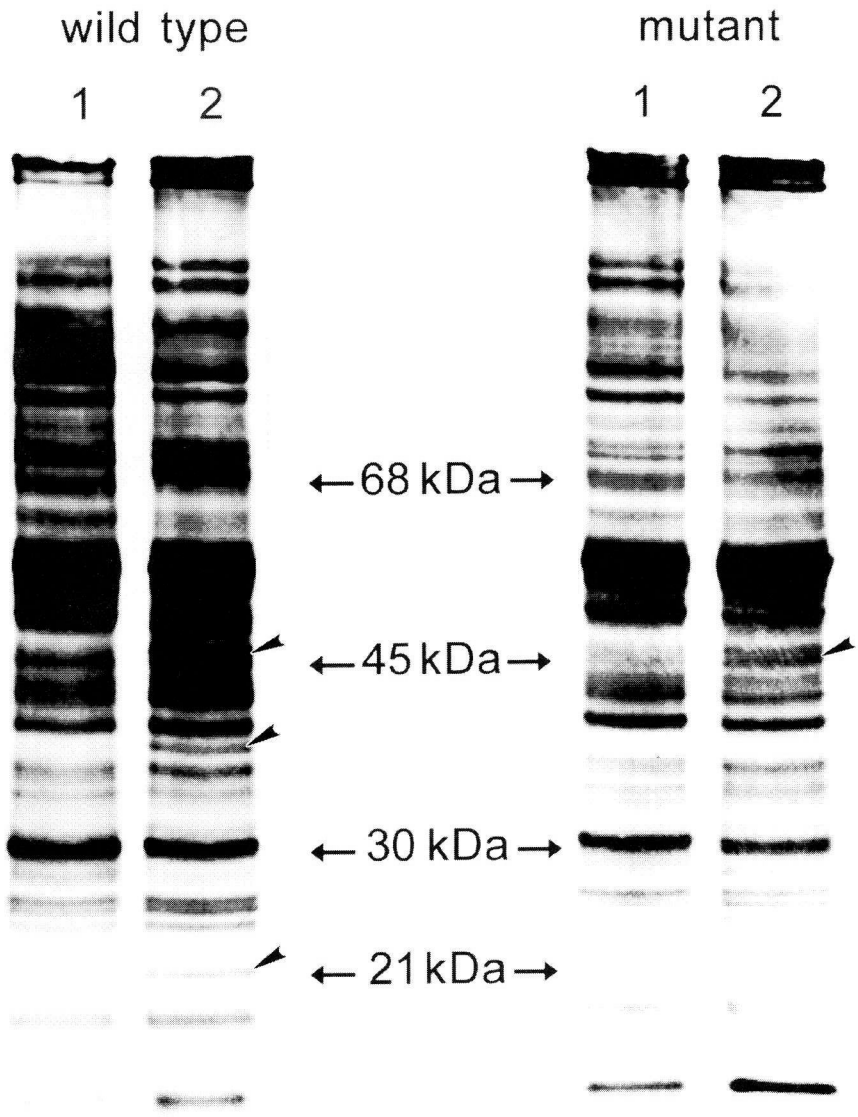

FIG. 1. – Autoradiography of ^{35}S-labelled protein analysis of *C. reinhardtii* wild-type and the *pyr*-45 cells. The labelled cell extracts were subjected to gradient 10-20% SDS-PAGE analysis. In lane 1, high CO_2-grown cells; lane 2, low CO_2-grown cells. The arrows indicate polypeptides which were preferentially labelled in wild-type and mutant cells on low CO_2.

Western blot protein analysis

Immunoblot analysis of wild-type total homogenates probed with antibodies raised against *C. reinhardtii* periplasmic CA showed reaction with a 37 kDa protein from low CO_2-grown cells only. Low CO_2 *pyr*-45 cells showed no such reaction (Fig. 2).

Immunoblot analysis of the total homogenates probed with antibodies raised against LIP-21 (Fig. 4) showed reaction with the 21 kDa polypeptide that appeared in low CO_2-grown wild type. No reaction was observed in *pyr*-45 total homogenates.

DISCUSSION

C. reinhardtii can grow photoautotrophically on very low levels of CO_2 due to the presence of a CCM. The CCM is inducible since only cells grown on low CO_2 exhibited a high apparent affinity for CO_2 (Badger *et al.*, 1980). The mechanism of the algal cells adaptation to low CO_2 conditions represents a process with a fairly complex organization and has not been characterized in detail. Several cell compartments are involved and inhibition of the activity (or its loss after mutations) of one or more enzymes leads to the malfunction of the mechanism

that governs adaptation of the photosynthesizing cell to conditions of CO_2 limitation, thus producing cells unable to adapt to low DIC conditions (Spalding *et al.*, 1983a,b,c; Badger and Price, 1992).

The appearance of low CO_2 inducible proteins is correlated with the induction of the CCM and these polypeptides have been suggested as participants in the mechanism or its induction (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Geraghty *et al.*, 1990; Spalding *et al.*, 1991; Ramazanov *et al.*, 1994a,b). Simultananeosly with the increase of the affinity of *C. reinhardtii* cells for external inorganic carbon, at least five polypeptides are induced (Coleman and Grossman, 1984a,b; Manuel and Moroney, 1988; Geraghty *et al.*, 1980; Mason *et al.*, 1990; Moroney and Mason, 1991; Spalding *et al.*, 1991). Actually, $^{35}SO_4^{-2}$ labelling (Fig. 1) and our immunoblot protein analysis (Fig. 2 and Fig. 3) show the induction of a 21, 36, 37 and 42-45 kDa in wild-type cells under low CO_2 conditions. These same polypeptides in *C. reinhardtii* have been described previously by other authors (Coleman and Grossman, 1984a,b; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Geraghty *et al.*, 1990; Mason *et al.*, 1990; Spalding *et al.*, 1991). In the mutant *pyr*-45 the induction of only two polypeptides of 42-45 kDa is observed under low CO_2 conditions (Fig. 1). These 42-45 polypeptides are also present in high CO_2-grown cells, althought in small amount. These results indicate that the induction of these polypeptides in the wild-type and in *pyr*-45 cells represent an up-regulation in the synthesis of the polypeptides rather than *de novo* induction of new polypeptides. This is in agreement with results previously described for wild-type by Spalding *et al.* (1991).

In many microalgae the CCM, which is induced by low CO_2, involves an extracellular and an intracellular CA (Spalding *et al.*, 1983a,b,c; Aizawa and Miyachi, 1986; Moroney and Mason, 1991; Fukuzawa *et al.*, 1990; Palmqvist *et al.*, 1990; Sultemeyer *et al.*, 1993; Ramazanov and Cárdenas, 1994). The mutant *cia*-5 has been shown to lack 37 kDa periplasmic CA as well as all the other low CO_2 inducible proteins. *Cia*-5 cells never show increased affinity for CO_2 even when they are grown on limiting CO_2 concentrations (Moroney *et al.*, 1989). It has been suggested that *cia*-5 mutant strain is defective in some factor which may either sense the CO_2 concentration or be responsible for the induction of the transcription of low CO_2-inducible genes (Moroney *et al.*, 1989; Spalding *et al.*, 1991). Our

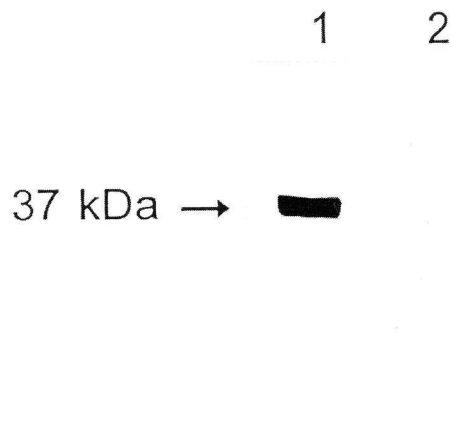

FIG. 2. – Immunoblot of the total homogenates from wild-type and mutant cells of *C. reinhardtii* probed with antibodies raised against the 37 kDa periplasmic carbonic anhydrase of *C. reinhardtii*. Lane 1, high CO_2 cells; lane 2, mutant cells. Each lane contained 100 µg of protein.

FIG. 3. – Immunoblot of the total cell homogenates from wild-type and the mutant cells probed with antibodies raised against LIP-21 kDa polypeptide. Lanes 1 and 2, wild-type cells; lane 1, high CO_2; lane 2, low CO_2; lanes 3 and 4, mutant cells; lane 3, high CO_2; lane 4, low CO_2. All lanes contained 100 µg of protein.

results show that, like *cia*-5 (Moroney *et al.*, 1989), *pyr*-45 induces neither LIP-36, LIP-21 nor the 37 kDa periplasmic CA proteins (Fig. 2). However, the *pyr*-45 mutant differs from *cia*-5, because the latter strain clearly senses the CO_2 conditions by increasing its affinity for DIC, although not to the level shown by the *C. reinhardtii* wild-type cells with induced CCM. Data for wild-type $K_{0.5}(CO_2)$ in our experiments (Table 1) were similar to those described for algae by others authors (Badger *et al.*, 1980; Spalding *et al.*, 1983a,b,c; Moroney *et al.*, 1989; Moroney and Mason, 1991). The addition of exogenous CA to low CO_2 *pyr*-45 cultures decreased the $K_{0.5}(CO_2)$ for photosynthesis from 10 to 6±1 µM

CO_2, still 3-fold greater than that of wild type. These results suggest that, in addition to the 37 periplasmic CA and 42-45 kDa proteins, the full functioning of the CCM requires other low CO_2-inducible proteins. Ramazanov *et al.* (1993) suggested that the 36 kDa polypeptide induced under low CO_2 conditions located in the chloroplast envelope may play an important functional role in the CCM. The actual role of all these polypeptides in this complex process still remains unclear.

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