A new chloroplast envelope carbonic anhydrase activity is induced during acclimation to low inorganic carbon concentrations in *Chlamydomonas reinhardtii*

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**Abstract** Using mass-spectrometric measurements of $^{18}$O exchange from $^{13}$C$^{18}$O$_2$ we determined the activity of carbonic anhydrase (CA; EC 4.2.1.1) in chloroplast envelope membranes isolated from *Chlamydomonas reinhardtii* cw-15. Our results show an enrichment of CA activity in these fractions relative to the activity in the crude chloroplast. The envelope CA activity increased about 8-fold during the acclimation to low-CO$_2$ conditions and was completely induced within the first 4 h after the transfer to air levels of CO$_2$. The CA activity was not dissociated from envelope membranes after salt treatment. In addition, no cross-reactivity with other CA isoenzymes of *Chlamydomonas* was observed in our chloroplast envelope membranes. All these observations indicated that the protein responsible for this activity was a new CA isoenzyme, which was an integral component of the chloroplast envelopes from *Chlamydomonas*. The catalytic properties of the envelope CA activity were completely different from those of the thylakoid isoenzyme, showing a high requirement for Mg$^{2+}$ and a high sensitivity to ethoxyzolamide. Analysis of the integral envelope proteins showed that there were no detectable differences between high- and low-inorganic carbon (C$_i$) cells, suggesting that the new CA activity was constitutively expressed in both high- and low-C$_i$ cells. Two different high-C$_i$-requiring mutants of *C. reinhardtii*, cia-3 and pmp-I, had a reduced envelope CA activity. We propose that this activity could play a role in the uptake of inorganic carbon at the chloroplast envelope membranes.

**Keywords** Carbonic anhydrase · *Chlamydomonas* (carbonic anhydrase) · Chloroplast envelope · CO$_2$-concentrating mechanism · Photosynthesis · Proteome

**Abbreviations** BTP: 1,3-bis[tris(hydroxymethyl)methyl-amino]propane · CA: carbonic anhydrase · CCM: CO$_2$-concentrating mechanism · C$_i$: inorganic carbon (CO$_2$ + HCO$_3^-$) · EZ: ethoxyzolamide · $I_{50}$: concentration of an inhibitor that causes a 50% reduction in the catalytic activity of a given amount of an enzyme · LIP: low-CO$_2$-inducible polypeptide · mtCA: (mitochondrial) carbonic anhydrase · pCA: (periplasmic) carbonic anhydrase

**Introduction**

The unicellular green alga *Chlamydomonas reinhardtii*, as well as other green algae, induces a CO$_2$-concentrating mechanism (CCM) when cells grown under high-CO$_2$ conditions (5% CO$_2$) are transferred to low-CO$_2$ conditions (ambient air) (Badger 1987; Badger and Price 1992; Spalding 1998; Moroney and Somanchi 1999). This mechanism allows algal cells to elevate the CO$_2$ concentration at the active site of Rubisco, ensuring efficient photosynthesis even under CO$_2$-limiting conditions. Several lines of evidence indicate that chloroplasts play an essential role in the operation of the CCM. First, active inorganic carbon (C$_i$) transport at the chloroplast envelope has been proposed by a number of researchers (Beardall 1981; Sültemeyer et al. 1988; Goyal and Tolbert 1989; Moroney and Mason 1991). In addition, it has been shown that chloroplasts isolated from low-C$_i$ *C. reinhardtii* cells accumulated C$_i$ to a higher extent than chloroplasts isolated from high-C$_i$ cells (Moroney et al. 1987). Using a mass-spectrometric technique it has
been recently shown that the predominant location of the C4 transport systems, both for CO2 and HCO3−, is at the chloroplast envelope (Amoroso et al. 1998). Second, chloroplastic CA activities increase up to 10-fold during acclimation to low-CO2 conditions (Ramazanov and Cárdenas 1992; Sültetemeyer et al. 1995; Amoroso et al. 1996). Third, low-CO2-inducible polypeptides (LIPs) are specifically localized to the chloroplast envelope membranes from C. reinhardtii (Ramazanov et al. 1993) and Dunaliella tertiolecta (Thielmann et al. 1992), although the functional role of these proteins has not been elucidated yet. All these observations point to the fact that the role of chloroplast envelope membranes in the process of C4 acquisition is more complex than expected.

The nature of the C4 uptake systems at the chloroplast envelope of green algae remains unknown. Amoroso et al. (1998) have reported the presence of inductive high-affinity CO2 and HCO3− transporters at the chloroplast envelope membranes from C. reinhardtii and Dunaliella tertiolecta. However, the proteins responsible for these transporters have not been identified yet. Recently, Rolland et al. (1997) have identified a new protein, called Ycf10, localized at the inner chloroplast envelope of C. reinhardtii. At present, Ycf10 is the only chloroplast envelope protein that has been related to an efficient C4 uptake into chloroplasts. However, there is presently no evidence as to whether this protein may be involved directly in this process or whether it may play an indirect role through pH regulation (Rolland et al. 1997). A vanadate-sensitive ATPase has been also suggested to be involved in the C4 transport process at the chloroplast level (Goyal and Tolbert 1989). Furthermore, some researchers have hypothesized that CO2 transport across the chloroplast envelopes could involve a CA-like polypeptide, as has been suggested for cyanobacteria (Bedu et al. 1992; Sültetemeyer et al. 1995; Price et al. 1998).

Another key element of the CCM that is localized to the chloroplast is CA (Badger and Price 1994; Sültetemeyer et al. 1995). Using mass-spectrometric measurements of 18O exchange, Sültetemeyer et al. (1995) have recently characterized two chloroplastic CA activities in C. reinhardtii cells that are induced during acclimation to low-CO2 conditions, an insoluble form associated with the thylakoid fraction, and a soluble form of CA activity. These authors have provided further evidence that these CA activities differ in their response to MgSO4 and ethoxylazolamide (EZ) (Amoroso et al. 1996). Support for the presence of the insoluble chloroplastic CA activity is provided by the identification of a 29.5-kDa α-type CA which is associated with the thylakoid membranes and constitutively expressed regardless of the CO2 concentration in the environment (Karlsson et al. 1995, 1998; Funke et al. 1997). However, there is little and controversial information about the occurrence of yet one more CA polypeptide in the chloroplast of this green alga. In this respect, Husic and Marcus (1994) reported on 21.5-and 26-kDa polypeptides that are specifically labeled by a CA-specific photoaffinity label-

ing technique. While it is likely that the 21.5-kDa protein is the mitochondrial CA (mtCA), the exact location of the 26-kDa protein is still to be determined. In this study we present evidence for the characterization of a new CA activity associated with the chloroplast envelope in C. reinhardtii cells, which is induced during acclimation to low-CO2 conditions.

Materials and methods

Algal strains and culture conditions

The cell-wall-deficient mutant of Chlamydomonas reinhardtii, CC-400 cw-15 mt− was obtained from the Chlamydomonas Culture Collection (Duke University, Durham, N.C., USA). The cia-3/cw-15 double mutant was a kind gift from Dr. J.V. Moroney (Louisiana State University, Baton Rouge, USA). The high-CO2-requiring mutant pmp-1 was kindly provided by Prof. M. Spalding (Iowa State University, Ames, USA). All strains were synchronously grown in high-salt medium (Sueoka 1960) at 28 °C, illuminated with a photosynthetic photon flux density (PPFD) of 200 μmol m−2 s−1, obtained from fluorescent tubes (TDL 150 W; Philips, Eindhoven, The Netherlands), with 12 h light/12 h dark cycles. Cells suspensions were aerated with either a CO2-air mixture (5:95, v/v; high-C4 cells) or with air (0.03% CO2; low-C4 cells). To obtain air-acclimated cells, high-C4-grown algae were collected by centrifugation, resuspended in fresh culture medium, and aerated with air for 4 h.

Preparation of protoplasts

To obtain protoplasts from the pmp-1 mutant strain, cells were harvested, washed once with 25 mM Bis-Tris-propane/HCl buffer (BTP-HCl, pH 8.0) and resuspended in 30 ml of autolysin (Sültetemeyer et al. 1989, 1990). After 30–45 min of incubation, protoplasts were centrifuged at 10,000 g for 5 min and washed six times with 25 mM BTP-HCl buffer (pH 8.0). The protoplast suspension was finally resuspended in the same buffer to yield a chlorophyll concentration of about 500 μg ml−1.

Isolation of chloroplasts and chloroplast envelopes

Chloroplasts from wild-type and mutant cells were isolated from protoplasts using digitonin, according to Sültetemeyer et al. (1995). Chloroplast envelopes were isolated as described by Douce and Joyard (1982). Briefly, purified intact chloroplasts (10–20 mg of chlorophyll) were lysed in hypotonic medium [50 mM Hepes-KOH (pH 7.8), 2 mM MgCl2; buffer A]. The suspension was homogenized vigorously with a Potter-Elvejem homogenizer. The lysate was loaded onto discontinuous sucrose gradients (0.3 M/1 M sucrose prepared in buffer A). Chloroplast envelope membranes were purified by centrifugation at 50,000 g for 1 h at 4 °C in an SW-40 swing-out rotor (L7 Ultracentrifuge; Beckman). After centrifugation, envelope membranes were collected from the 0.3/1 M sucrose interface and washed twice with 10 mM Tris-HCl buffer (pH 7.8), 1 mM EDTA. Finally, envelope membranes were pelleted at 120,000 g for 1.5 h and resuspended in the same buffer. When chloroplast envelope membranes were used for CA determination, these fractions were resuspended in 25 mM BTP-HCl buffer (pH 8.0).

Differential extraction of chloroplast envelope proteins

Two different methods were used for extracting envelope proteins. When CA activity was to be determined in the different extracted fractions, chloroplast envelope membranes were resuspended in 12.5 mM Tris-HCl buffer (pH 8.2), containing 300 mM KCl. The