Discovery of an algal mitochondrial carbonic anhydrase: Molecular cloning and characterization of a low-CO2-induced polypeptide in *Chlamydomonas reinhardtii*

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**ABSTRACT** In green unicellular algae, several polypeptides are induced upon exposure to limiting CO2. We report here on the localization and characterization of one of these, a 22-kDa polypeptide in *Chlamydomonas reinhardtii*. This nuclear-encoded polypeptide is induced in the mitochondria by a lowering of the partial pressure of CO2 in the growth medium from 5% to air CO2 levels. Sequencing of two different cDNA clones coding for the polypeptide identified it as a 20.7-kDa β-type carbonic anhydrase (CA; carbonate dehydratase, carbonate hydro-lyase, EC 4.2.1.1). The two clones differ in their nucleotide sequences but code for identical proteins, showing that this CA is encoded by at least two genes. Northern blot hybridization reveals that mRNA transcripts are only present in cells transferred to air CO2 levels. A comparison of the deduced amino acid sequence with those of other β-CAs shows the largest degree of similarity with CA from the cyanobacterium *Synechocystis* (50% identity and 66% similarity). To our knowledge, this is the first identification and characterization of a mitochondrial CA from a photosynthetic organism.

Carbonic anhydrase (CA; carbonate dehydratase, carbonate hydro-lyase, EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the interconversion of CO2 and HCO3-. It is involved in many different processes, such as ion exchange, acid-base balance, carbon dioxide fixation, and organic carbon diffusion between the cell and its environment. A variety of CA isoenzymes are found in animal, plant, and archea green algae, and animals. The β-CAs (eubacteria and plant chloroplasts) and γ-CAs (archaeabacteria, euabacteria, and plants). In higher vertebrates, seven α-CA isoenzymes have been described. One of these, CA V, is located in the mitochondria and has been proposed to operate in the mechanism of CO2 entry to the urea cycle to gluconeogenesis.

In unicellular green algae and cyanobacteria, CA plays a role in the CO2 concentrating mechanism which maintains a high concentration of CO2 at the active site of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39), the major carboxylating enzyme in photosynthesis (8). The CO2 concentrating mechanism is induced in these organisms as an adaptation to low-CO2 conditions. In the green microalga *Chlamydomonas reinhardtii*, the induction of at least seven polypeptides is associated with the appearance of the members of the cells to actively accumulate inorganic carbon (9–11). One of these, a 37-kDa polypeptide, has been identified as an extracellular α-CA (12, 13). Another one has been localized to the chloroplast envelope (14), but the function of that 36-kDa polypeptide is not known. Recently, Chen et al. (15) reported that the gene encoding alanine aminotransferase is induced in low CO2 and codes for a 55-kDa polypeptide. The identification, characterization, and localization of the other four to seven reported low-CO2-induced polypeptides have not been reported.

We are examining the role of the mitochondria in the CO2 concentrating mechanism in *C. reinhardtii*. In this report we show that one of the polypeptides induced by low CO2 is a 20.7-kDa β-CA located in the mitochondria. We have sequenced two different cDNA clones coding the protein and both of them are expressed only under low-CO2 conditions. To our knowledge, this is the first evidence of a mitochondrial CA in a photosynthetic organism. We propose that its function is to act as a pH stabilizer and to facilitate diffusion of CO2 out of the mitochondrial matrix under low-CO2 (high photosynthetic conditions); this CO2 is produced by glycine decarboxylation in the photosynthetic pathway.

**MATERIALS AND METHODS**

**Algal Strain and Culture Conditions.** The green alga *C. reinhardtii* mutant strain CW 92 was grown in batch cultures at 25°C with continuous light at an intensity of 150 µmol quanta m-2s-1. Bottles containing 2 liters of minimal medium were vigorously bubbled with air containing 5% CO2. To obtain low-CO2-adapted cultures, the cultures were switched to air 10 h prior to isolation of mitochondria. The major components of the medium were prepared according to Sueoka (16), and the trace element solution was prepared according to Hutner et al. (17).

**Isolation of Mitochondria.** Mitochondria were isolated as reported (18), except that the bovine serum albumin was omitted from all solutions.

**35S-Labeling.** Algae were grown in a medium containing one-tenth of the normal sulfur content and bubbled with air containing 5% CO2. On the third day of growth, the algae were pelleted and washed twice in 20 mM Hepes/ KOH (pH 7.2). The algae were resuspended in growth medium without sulfur. Half of the culture was bubbled with air and half with air containing 5% CO2. Carrier-free 35SO42- was added (2.5 mCI/liter; 1 Ci = 37 GBq) 30 min after the transfer. Mitochondria were isolated 10 h after labeling.

**Polyacrylamide Gel Electrophoresis.** SDS/PAGE (12.5% polyacrylamide) was performed as described by Laemmli (19) using the Mini-Protein II (Bio-Rad) slab-gel apparatus. The molecular weight markers used for the silver-stained gel were α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase B (94 kDa).

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**Immunoblotting.** The polypeptides were electroblotted from a SDS/PAGE gel to nitrocellulose filters for immunodetection with antisera directed against Rubisco from *Spinacia oleracea*. The secondary antibodies were conjugated to alkaline phosphatase. The methods used were described by Harlow and Lane (20).

**Cytochrome c Oxidase Activity, Chlorophyll, and Protein Determinations.** Cytochrome c oxidase activity was assayed as described by Wigge and Gardeström (21). Chlorophyll was extracted in cold methanol, and the concentration was calculated according to Porra et al. (22). The protein content was measured according to Peterson (23), using bovine serum albumin as the standard protein.

**Screening of cDNA Library.** To obtain N-terminal and internal amino acid sequences, the polypeptide was analyzed as reported by Karlsson et al. (24). Using the *C. reinhardtii* codon usage, two degenerate oligonucleotides were designed and synthesized with parts of two of the internal amino acid sequences as templates (VNNKPHD and VQMELK). Inosine was used where codon usage was highly degenerate and the 5'-ends were extended with *EcoRI* restriction sites [p1, 5'-GCCGAATTTCGTTA(A/C/T)AA(C/T)AGCCICA(C/T)GA-3'; and p2, 5'-GCCGAATTTCGTTCA(G/A)ATGGAGAC(A/G)CTTAA-3']. PCRs with both designed primers against the vector-specific T7 primer were run for 1 min at an annealing temperature of 52°C and with *Taq* polymerase (Perkin-Elmer) for extension. Total DNA from a cDNA library was used as template [Uni-ZAP XR library kit; Stratagene (25)]. The p1/T7 primer combination gave a 1050-bp product and primer p2/T7 primer gave a 750-bp product. The 750-bp PCR product was also obtained when the 1050 PCR product was used as template, verifying the common origin of the two products. A 950-bp *EcoRI*-KpnI fragment from the 1050-bp PCR product was used to screen the Uni-ZAP XR library. Two positive clones were identified and sequenced.

**Northern Blot Analyses.** The two clones had slightly different nucleotide sequences. To determine if both of them were induced by low CO₂, Northern blot analyses were done with probes (20 and 27 bp) binding to a region in the 3'-untranslated region that differed between the two clones (see Fig. 2). The specificities of the probes were confirmed by probing against the sequenced clones (results not shown). Total nucleic acid was isolated according to Johanningmeier and Howell (26) and RNA was purified with RNeasy Total RNA Kit (Qiagen, Chatsworth, CA). The glyoxal-denatured northern blots were done according to Sambrook et al. (27), but using 6× standard saline citrate (0.9 M NaCl/0.09 sodium citrate, pH 7.0). Hybridization and washing were done at 42°C.

**RESULTS AND DISCUSSION**

**Low-CO₂-Induced Mitochondrial Polypeptides.** SDS/PAGE gels of mitochondria isolated from high- and low-CO₂-grown cells reveal that at least three polypeptides with electrophoretic mobilities corresponding to 22, 24, and 36 kDa are induced in the mitochondria of low-CO₂-grown cells (Fig. 1A). The 24- and 36-kDa polypeptides are less abundant than the 22-kDa polypeptide, and at the moment we have no data showing the identity of these polypeptides. The 22-kDa polypeptide is of a similar size as previously reported for low-CO₂-induced polypeptides (9–11). Induction of the 22-kDa polypeptide is also demonstrated on autoradiograms of mitochondria isolated from 35S-labeled algae (Fig. 1B). The 22-kDa polypeptide is one of the most abundant polypeptides after the transfer to low-CO₂ conditions.

In Table 1, data related to the purity of the isolated mitochondria are presented. The mitochondrial enzyme cytochrome c oxidase is enriched about 10 times and the contamination of thylakoid membranes is less than 4%. The cytochrome c oxidase is purified about 500 times over chlorophyll (thylakoid membranes). Fig. 1C demonstrates that the mitochondrial fraction is free also from contaminating soluble proteins (and intact chloroplasts), since no immunoreaction with antiserum against Rubisco is seen. The soluble protein Rubisco is present in large amounts in the cell and is known to contaminate most subcellular fractions. These results show that the enrichment of the mitochondria is sufficient to allow the conclusion that the 22-kDa polypeptide is mitochondrial.

**Sequence Characteristics.** The two sequenced clones represent two different cDNAs (96% identity) (Fig. 2). There are four possible start codons, giving transit-peptides of 70, 59, 44, or 43 amino acids. At the moment we have no data identifying the correct one. In the coding region the two cDNA clones differ at seven nucleotides. Only one of these differences results in a difference in the amino acid sequence, a serine to alanine shift at position 53 in the presequence, so the mature proteins encoded by the two cDNAs are identical. Additional nucleotide differences are located in the 3'-untranslated region.

*Fig. 1.* SDS/PAGE of isolated *C. reinhardtii* mitochondria. (A) Silver stained gel (15 µg protein per lane). (B) Autoradiogram of a gel with separated mitochondrial polypeptides isolated from 35S-labeled cells (equivalent amount of radioactivity in both lanes). (C) Immunoreaction against Rubisco antiserum with cell homogenate and isolated mitochondria from low-CO₂-grown cells (15 µg protein per lane). +, Mito. low-CO₂, mitochondria isolated from low-CO₂-grown cells; †, Mito. high-CO₂, mitochondria isolated from high-CO₂-grown cells.

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**Table 1. Purification of mitochondria**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cyt c ox, µmol·mg⁻¹ protein⁻¹</th>
<th>Chl, nmol·mg⁻¹</th>
<th>Cyt c ox/Chl, relative units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High CO₂</td>
<td>Low CO₂</td>
<td>High CO₂</td>
</tr>
<tr>
<td>Homogenate</td>
<td>3 ± 2</td>
<td>2 ± 1</td>
<td>208 ± 57</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>23 ± 7</td>
<td>23 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

Distribution of markers for mitochondria (cytochrome c oxidase, Cyt c ox) and thylakoids (chlorophyll, Chl) after fractionation of *C. reinhardtii* grown in high and low CO₂. All values are means ± SD of three preparations.

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Identification of the 22-kDa Polypeptide. The deduced amino acid sequence is similar to those of the previously sequenced β-CAs (Fig. 3). It contains most of the amino acids that are highly conserved in the β-CAs family, including the
three putative zinc ligands (28). In accordance with the terminology used for other CA genes, these genes are called β-CA1 and β-CA2. To our knowledge, this is the first report of a β-CA in C. reinhardtii. Earlier three α-CAs have been reported. Two of these are periplasmic proteins (29) and, recently, Karlsson et al. (24) reported an intracellular α-CA with a proposed localization in the chloroplast. A detectable activity of CA induced by low CO₂ was measured in the mitochondrial fractions using an electrochemical detection method (data not shown). The activity was Triton X-100 dependent, indicating that the CA is localized inside the mitochondria, and is therefore not likely to be due to contaminating extracellular CA. On the amino acid sequence level, the mitochondrial CA from C. reinhardtii is more similar to bacterial β-CAs and to another β-CA from a green alga (Chlamydomonas sp., 30) than to β-CAs from higher plants (Fig. 3B). The highest degree of similarity is found with a proposed CA from the cyanobacterium Synechocystis sp. PCC6803 (31), 50% identity and 66% similarity. The mitochondrial CA has a lower molecular weight than the other β-CAs due to six small gaps in the sequence, a feature shared with the putative CA from Synechocystis, and a shorter C-terminal (Fig. 34). When Husic and Marcus (32) used a CA-directed photoaffinity reagent they detected weak labeling of a 21.5-kDa polypeptide, but they concluded that the weak signal was not enough to suggest that the polypeptide was a CA without further experiments.

The Expression of β-CA1 and β-CA2. Northern blot analyses were performed to determine if both genes are induced or if one of them is expressed constitutively. Using gene-specific probes, we observed that both genes are induced by low CO₂ and that neither of them is expressed at high CO₂ (Fig. 4). In Fig. 1 it can be seen that a small amount of a 22-kDa polypeptide is also present in high CO₂ grown cells. Since no transcription products of the β-CA1 or β-CA2 genes were seen at high CO₂, we suggest this is another mitochondrial polypeptide of the same molecular size as the CA.

Role and Function of an Algal Mitochondrial CA. When an algal cell grown at high CO₂ is transferred to low CO₂, the photosynthetic rate initially increases (33, 34). After 5–10 h, when the CO₂ concentrating mechanism is fully induced, photosynthesis decreases due to the higher intracellular CO₂ concentration. Photosynthetic glycine decarboxylation produces equivalent amounts of CO₂ and NH₃ (35). Since NH₃ at the pH of the mitochondrial matrix will form NH₄⁺, H⁺ is consumed. This H⁺ uptake is likely to be considerably faster than the hydration of CO₂ when H₂O is produced. This could result in an alkalinization of the mitochondrial matrix at the onset of photorespiration. A possible role for a mitochondrial CA might thus be to function as a pH stabilizer to prevent such an alkalinization. A role for CA in pH regulation has been suggested in other systems (36). A mitochondrial CA could also be involved in the diffusion and transport of inorganic carbon species within the mitochondrial matrix as well as their efflux, in analogy to what has been shown in other systems (3, 37).

The level of induction of this CA indicates that it probably has an important role in adaptation of this algal cell to low-CO₂ conditions. This study provides the basis for experimental studies of the function of this mitochondrial CA.

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