A novel α-type carbonic anhydrase associated with the thylakoid membrane in Chlamydomonas reinhardtii is required for growth at ambient CO₂

Jan Karlsson  
Umeå Universitet

Adrian K. Ciarke  
Umeå Universitet

Zhi Yuan Chen  
Louisiana State University

Stephanie Y. Huggins  
Louisiana State University

Youn Il Park  
Umeå Universitet

See next page for additional authors

Follow this and additional works at: https://digitalcommons.lsu.edu/biosci_pubs

Recommended Citation
Authors
Jan Karlsson, Adrian K. Ciarke, Zhi Yuan Chen, Stephanie Y. Hugghins, Youn Il Park, H. David Husic, James V. Moroney, and Göran Samuelsson

This article is available at LSU Digital Commons: https://digitalcommons.lsu.edu/biosci_pubs/3113
A novel α-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO₂

**Jan Karlsson**, Adrian K.Clarke, Zhi-Yuan Chen, Stephanie Y.Hugghins, Youn-II Park, H.David Husic, James V.Moroney and Göran Samuelsson

Department of Plant Physiology, Umeå University, S-901 87 Umeå, Sweden; 2Department of Plant Biology, Louisiana State University, Baton Rouge, LA 70808 and 3Department of Chemistry, Lafayette College, Easton, PA 18042, USA

1Corresponding author
e-mail: jan.karlsson@plantphys.umu.se

A 29.5 kDa intracellular α-type carbonic anhydrase, designated Cah3, from the unicellular green alga *Chlamydomonas reinhardtii* is the first of this type discovered inside a photosynthetic eukaryote cell. We describe the cloning of a cDNA which encodes the protein. Immunoblot studies with specific antibodies raised against Cah3 demonstrate that the polypeptide is associated exclusively with the thylakoid membrane. The putative transit peptide suggests that Cah3 is directed to the thylakoid lumen, which is confirmed further by the presence of mature sized Cah3 after thermolysin treatment of intact thylakoids. Complementation of the high inorganic carbon concentration-requiring mutant, cia-3, with a subcloned cosmid containing the cah3 gene yielded transformants that grew on atmospheric levels of CO₂ (0.035%) and contained an active 29.5 kDa α-type carbonic anhydrase. Although, cia-3 has reduced internal carbonic anhydrase activity, unexpectedly the level of Cah3 was raised against Cah3 demonstrate that the polypeptide contained an active 29.5 kDa α-type CA, with a subcloned cosmid containing the cah3 gene yielded transformants that grew on atmospheric levels of CO₂ (0.035%) and contained an active 29.5 kDa α-type carbonic anhydrase. Although, cia-3 has reduced internal carbonic anhydrase activity, unexpectedly the level of Cah3 was similar to that of the wild-type, suggesting that the mutant accumulates an inactive Cah3 polypeptide. Genomic sequence analysis of the mutant revealed two amino acid changes in the transit peptide. Results from photosynthesis and chlorophyll a fluorescence parameter measurements show that the cia-3 mutant is photosynthetically impaired. Our results indicate that the carbonic anhydrase, extrinsically located within the chloroplast thylakoid lumen, is essential for growth of *C.reinhardtii* at ambient levels of CO₂ and that at these CO₂ concentrations the enzyme is required for optimal photosystem II photochemistry.

**Keywords**: CCM/cia-3/complementation/thylakoid lumen/transfer peptide

**Introduction**

CO₂ and HCO₃⁻ are substrates and products of many different metabolic reactions in cells. The uncatalysed interconversion between CO₂ and HCO₃⁻ is slow, with a $k_{1/2}$ for the hydration reaction of 14.1 s (Smith, 1988). Carbonic anhydrase (CA; carbonic dehydratase, carbonate hydratase, EC 4.2.1.1) catalyses the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$ with a maximum turnover number in excess of $10^6/s$ (Khalifah, 1971). To date, three phylogenetically independent gene families encoding distinct types of CAs have been identified (α-, β- and γ-CA; reviewed by Hewett-Emmett and Tashian, 1996) that share no significant sequence homology. CAs are involved in a variety of biological processes including pH regulation, respiration and photosynthesis (Tashian, 1989; Badger and Price, 1994).

Most is known about the α- and β- types, whereas the γ-type CA has been identified only recently in archaebacteria. The α-CA is the only type found in animals, but it also occurs in the periplasmic space of the eukaryotic unicellular green alga *Chlamydomonas reinhardtii* and in the eubacteria *Neisseria gonnorhoeae*. The β-CAs were first discovered in the stroma of higher plant chloroplasts, but have since been found in eubacteria (Hewett-Emmett and Tashian, 1996) and in algal mitochondria (Eriksson *et al.*, 1996).

Aquatic plants must overcome the slow diffusion of CO₂ in water to photosynthesize effectively. The green alga, *C.reinhardtii*, and many other green algae and cyanobacteria have the ability to concentrate inorganic carbon (Ci) in response to varying external levels during growth. When cells are exposed to low Ci levels (in solutions equilibrated with ambient air containing 0.035% CO₂, for instance), a Ci-concentrating mechanism (CCM), is induced to actively supply CO₂ to Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39; Aizawa and Miyachi, 1986; Badger and Price, 1994). The synthesis of at least seven polypeptides is induced concomitantly with the CCM (Moroney *et al.*, 1985; Fukuzawa *et al.*, 1990; Rawat and Moroney, 1991), including a 37 kDa periplasmic α-CA (pCA, Fukuzawa *et al.*, 1990), a 36 kDa chloroplast envelope polypeptide (Ramazanov *et al.*, 1993), a 53 kDa polypeptide identified as an alanine-aminotransferase (Chen *et al.*, 1996) and, recently, a 21 kDa mitochondrial β-CA (mtCA, Eriksson *et al.*, 1996).

The specific function of every one of the seven inducible polypeptides is so far unknown, although pCA is proposed to accelerate the equilibration of the predominant Ci species, HCO₃⁻ and CO₂, outside the plasma membrane (Moroney *et al.*, 1985), thereby allowing CO₂ to enter the cell at a rate sufficient to support photosynthesis.

It has been inferred that CAs are also present within algal cells, as they are in higher plant cells, in the chloroplast and/or the cytoplasm (Spalding *et al.*, 1983; Moroney and Mason, 1991; Sültemeyer *et al.*, 1995; Amoroso *et al.*, 1996). CA activity has also been found associated with, or in components tightly bound to, thylakoid membrane preparations from higher plants (Komarova *et al.*, 1982) and green algae (Pronina and Semenenko, 1988; Pronina and Borodin, 1993). This activity is thought to be involved either in the regulation of photosynthesis at low Ci levels or, more likely, in the regulation of intracellular pH (Tashian, 1989; Badger and Price, 1994).
of photosynthetic electron transport (Moubarak-Milad and Stemler, 1994) or as an important component of the CCM (Raven, 1997). We recently reported the purification of a 29.5 kDa intracellular α-CA from *C.reinhardtii* which is distinct from the pCA (Karlsson *et al*., 1995). A number of mutants have also been isolated that apparently lack an internal CA and require elevated levels of CO₂ for growth (Spalding *et al*., 1983; Moroney *et al*., 1986). One of these mutants, *cia*-3, fails to label a protein with a CA-specific photoaffinity label (Husic and Marcus, 1994) which in wild-type cells reacts with a 30 kDa protein. Here we report the identification of an intracellular α-type CA exclusively associated with the thylakoid membrane and targeted to the chloroplast lumen. We describe the cloning of a full-length cDNA clone, designated *cah3*, encoding this protein. We also show that expression of the *cah3* gene can complement the phenotype of the *cia*-3 mutant, restoring its intracellular CA activity and photosynthetic capacity, thereby allowing the transformants to grow in low CO₂ environments. We believe these results are of fundamental importance to the study of the CCM in algae and of photosynthesis in general, because they describe for the first time molecular evidence for a thylakoid-associated CA.

**Results**

**Isolation and sequence of *cah3* cDNA**

Tryptic fragments obtained after cleavage of an intracellular 29.5 kDa CA polypeptide previously isolated from *C.reinhardtii* (Karlsson *et al*., 1995) were used to design degenerate oligonucleotide primers. A probe was obtained from a cDNA library, via PCR, and that was used to screen the same library for full-length cDNA clones. Several positive clones were identified and sequenced. The longest of these cDNA clones was 1383 bp and contained an open reading frame encoding a polypeptide of 310 amino acids (Figure 1A). The cDNA also had a 61 nucleotide 5′-untranslated region and a 389 nucleotide 3′-untranslated region (excluding the polyadenylated tail). A polyadenylation signal (TGTTAA) characteristic of nuclear-encoded genes in *C.reinhardtii* (Silflow *et al*., 1985) was located 372 bp downstream of the stop codon (Figure 1A). Subsequent genomic Southern hybridization indicated that *cah3* is a single copy gene (results not shown). The calculated molecular mass of the mature polypeptide is 26 058 Da, with a predicted isoelectric point of 7.87. The putative start codon is 72 amino acids upstream of the mature N-terminus, as previously determined by direct sequencing of isolated Cah3 protein (Karlsson *et al*., 1995). When the deduced amino acid sequence of the mature Cah3 polypeptide was compared with other known CAs, the highest degree of similarity was found to those of the α-type (Figure 1B). Overall, Cah3 shares 30–40% identity with other α-CAs, with up to 90% identity within the conserved domains characteristic of this CA type. It is most similar to the α-CA from the bacterium, *N.gonorrhoeae*, with which it shares 40.6% identity. Within the conserved domains, Cah3 possesses 26 of the 36 amino acid residues with side chains that project into or border the active site cavity in mammalian α-CAs (Hewett-Emmett and Tashian, 1996; Figure 1A). Furthermore, the three histidine residues that function as Zn ligands within the active site of all α-CAs are also present in Cah3 (Figure 1A).

The 72 amino acid pre-sequence of Cah3 contains sequence elements that are characteristic of proteins targeted into the thylakoid membrane lumen. The lumen-targeting transit peptides in *C.reinhardtii* are bipartites of an N-terminal stroma-targeting domain and a C-terminal lumen-targeting domain (Franzén *et al*., 1990). The stromal-targeting domain is cleaved by a stromal peptidase at a semiconserved cleavage site, Val-X-Ala, proposed to be Val-Arg-Ala in Cah3 (Figure 1C). The lumen-targeting domain contains a hydrophobic region followed by a conserved lumen peptidase cleavage site, Ala-X-Ala. In Cah3, the hydrophobic region is followed by Ala-Lys-Ala (Figure 1C).

In higher plants, some polypeptides targeted to the lumen are translocated by a ΔPH-dependent process, which is distinct from the Sec-related processes. Figure 1C shows a comparison of the transit peptides of Cah3 from wild-type *C.reinhardtii* and the *cia*-3 mutant, together with the *C.reinhardtii* oxygen-evolving enhancer proteins, OEE2 and OEE3, the barley photosystem I reaction centre subunit psaN (PSI-N) and the tomato polyphenol oxidase A (PPO) transfer peptides. The corresponding higher plant OEE2 and OEE3 polypeptides from wheat (23 kDa) and spinach (16 kDa) respectively, and the barley PSI-N and tomato PPO have all been shown to be imported by the ΔPH-dependent translocation pathway (Nielsen *et al*., 1994; Chaddock *et al*., 1995). A comparison of these higher plant transfer peptides shows a high degree of similarity, with a characteristic twin arginine motif in the lumen-targeting domain, closely followed by the hydrophobic region (Chaddock *et al*., 1995). It is therefore plausible that the *C.reinhardtii* OEE2, OEE3 and Cah3 polypeptides are translocated via the same trans-thylakoid proton gradient mechanism, consistent with the hypothesis that Cah3 is directed to the thylakoid lumen. This hypothesis is strengthened further by the presence of a lysine residue in the C-terminus of Cah3, a few residues before the conserved lumen peptidase cleavage site, which recently has been shown to be an important part of a ‘Sec-avoidance’ signal (Bosch *et al*., 1997).

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under Accession number U40871.

**Regulation of *cah3* by external CO₂ concentration during growth**

Since intracellular CA has been proposed to be involved in the CCM (Spalding *et al*., 1983), the expression of the *cah3* gene in wild-type (wt-137c) *C.reinhardtii* was measured upon changing CO₂ levels from 5% (high Cᵢ) to atmospheric levels (low Cᵢ; i.e. 0.035% CO₂) during cultivation. Northern blot hybridization of total RNA isolated from high and low Cᵢ-grown cells (Figure 2) demonstrated that the *cah3* gene is expressed under high Cᵢ as a transcript of 1.45 kb. The level of this transcript, however, approximately doubles after transfer of the culture to low Cᵢ relative to the amount of 16S/18S rRNA present. Induction of the CCM by this low Cᵢ treatment was confirmed by analysing the induction of the low Cᵢ-inducible 21 kDa CA located in the mitochondria (Eriksson *et al*., 1996); no transcripts for this gene were detected.

A novel α-type carbonic anhydrase in *C.reinhardtii*
under high $C_i$ but strong induction occurred after transfer to low $C_i$, indicating that the changed conditions had induced CCM.

Localization of Cah3 by immunoblot analysis

To examine the localization of Cah3 within the cell, specific antisera were raised against a synthetic peptide derived from an internal sequence of the $cah3$ gene product. As shown in Figure 3, the antibodies recognized a polypeptide of ~30 kDa in isolated chloroplasts from high and low $C_i$-grown $C. reinhardtii$ cells (lanes 3 and 4). Cah3 was located only in the chloroplast thylakoid fractions (lanes 7 and 8) and not in the stromal fraction (data not shown), nor in the fraction containing chloroplast envelope membrane proteins (lanes 5 and 6). Differences were not apparent in the amount of Cah3 protein in high and low $C_i$-grown cells, in contrast to the slight induction of $cah3$ transcripts observed following the shift to low $C_i$ conditions.

Identification of Cah3 in the CCM mutant, cia-3

Several mutants of $C. reinhardtii$ have been isolated that are apparently deficient in intracellular CA activity (Spalding et al., 1983; Moroney et al., 1986; Katzman et al., 1994). Previously, we described an active site-directed photoaffinity reagent, $[^{125}I]p$-aminomethylbenzene-sulfonamide-4-azidosalicylamide ($[^{125}I]PAMBS-ASA$), that upon photoactivation specifically covalently modifies...
and labels CA. Using this reagent, a 30 kDa polypeptide believed to be the chloroplastic CA was labelled in wild-type (wt-137c) cells but not in the CA-deficient mutant cia-3 (Husic and Marcus, 1994). We therefore transformed the double mutant strain cia-3/cw-15 with a cosmid (~40 kbp of genomic DNA) containing the cah3 gene and selected for cells that could grow on low Ci. Approximately 45 transformants were recovered from two separate transformations. In contrast to the original strain, most of these transformants could grow well on ambient CO2 (Figure 4). A subcloned 4 kbp piece of the genomic clone containing the cah3 gene was also able to complement the cia-3 phenotype, minimizing the possibility that another gene present on the large cosmid was responsible for the recovery of cia-3.

Following photoaffinity labelling, the 30 kDa polypeptide labelled in wild-type cells was observed in the transformants, but not in the original cia-3 or cia-3/cw-15 mutants (Figure 5). Furthermore, when the cia-3 strain was crossed with the wild-type, analysis of the resultant tetrad progeny (Table I; TD1–TD4) revealed co-segregation of the presence of the 30 kDa photoaffinity-labelled polypeptide and the efficient Ci utilization characteristic of wild-type cells. These results are consistent with there being a link between the loss of a putative internal CA and the growth requirement for high Ci. This indicates that the transformants express the gene encoding an active internal CA which enables them to grow on low Ci.

![Image](92x460_to_262x554)

**Fig. 2.** Northern hybridization analyses of wild-type (wt-137c) cells grown in high and low Ci. Comparison of *C. reinhardtii* total RNA (10 µg) isolated from high Ci (lane 1) and low Ci-grown cells (lane 2). Filter A was hybridized with the cah3-specific probe. Filter B was hybridized with the low Ci-inducible mitochondrial 21 kDa CA-specific probe. Denatured DNA size standards in kb (Gibco-BRL) are indicated on the left.

![Image](324x594_to_544x742)

**Fig. 3.** Immunoblot analysis of whole cell and chloroplast fractions. A comparison of whole cells (lanes 1 and 2), chloroplasts (lanes 3 and 4), envelope membranes (lanes 5 and 6) and thylakoid membranes (lanes 7 and 8). High Ci-grown cells and subcellular fractions were used for lanes 1, 3, 5 and 7. Low Ci cells and subcellular fractions were used for lanes 2, 4, 6 and 8. 150 µg of protein was loaded in whole cell lanes and 15 µg in the subcellular fraction lanes. Cah3-specific, custom-made antibodies were used. Molecular mass markers are shown on the left.

![Image](325x387_to_543x520)

**Fig. 4.** Growth of double mutant strain *cia-3/cw-15* and strains transformed with cosmids containing the *cah3* gene. Strains *cc-400*, *cia-3/cw-15* and four independent transformants (1b, 6a, 7a and 9a) were plated on minimal media and grown for 7 days under elevated CO2 conditions (High CO2) or under ambient air (Low CO2).

![Image](300x594_to_511x742)

**Fig. 5.** Photoaffinity labelling of *cia-3/cw-15* cells transformed with the cah3 gene. Extracts from the indicated cell lines were labelled with the CA-directed photoaffinity label [125I]PAMBS-ASA as in Husic and Marcus (1994). Specific labelling of the 30 kDa polypeptide (marked with an arrow) is judged by those proteins labelled in the absence (-) but not in the presence (+) of an excess of an unlabelled competing sulfonamide, ethoxyzolamide (Husic and Marcus, 1994). Samples were wild-type cells (wt-cc400), the cell wall-deficient mutant *cw-15* (cc400), the CA-deficient mutant *cia-3*, a *cia-3/cw-15* double mutant, and four strains of *cia-3/cw-15* transformed with the cah3 gene that were capable of growth on atmospheric levels of CO2 (1B, 6A, 7A and 9A). Molecular mass markers are shown on the left.

<table>
<thead>
<tr>
<th>Cell line</th>
<th><em>K</em>&lt;sub&gt;0.5&lt;/sub&gt;Ci (µM)</th>
<th>Photoaffinity-labelled 30 kDa</th>
<th>Immuno-reactive 30 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc-124</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cia-3</td>
<td>45</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TD1</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TD2</td>
<td>26</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TD3</td>
<td>2.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TD4</td>
<td>37</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Chlamydomonas reinhardtii* cells were grown at 5% CO2, and transferred to atmospheric levels of CO2, 5 h before harvesting. Photosynthesis rates at saturating levels of CO2 were >140 µmol/h/mg Chl for all strains. The presence of the 30 kDa polypeptide was determined by photoaffinity labelling of cell extracts with [125I]PAMBS-ASA and by immunoblot analyses with Cah3-specific antibodies.

**Table I.** Characterization of tetrads progeny (TD1–TD4) of a cross between the CCM mutant (*cia-3*) and wild-type (cc-124) *C. reinhardtii* cells
Moreover, internal CA activities measured in the transformants were found to be in the same range as that of the wild-type (80–120%).

Immunoblot analyses of total protein extracts from the cia-3 strain with the Cah3-specific antisera indicated, surprisingly, that the mutant had a level of Cah3 similar to the wild-type (Figure 6). This strongly suggests that the phenotype of this CCM mutant results from the production of an inactive Cah3 protein rather than the inactivation of Cah3 synthesis per se.

Sequencing of the genomic region of the cah3 gene in the cia-3 mutant revealed two point mutations in the transit peptide. These mutations, T→G (position 137; Figure 1A) and C→A (position 139), cause changes in the amino acid composition, with a leucine pair becoming arginine and methionine. As shown in Figure 1C, these changes occur at the beginning of the hydrophobic region close to the twin arginines that are known to be important for the ΔpH-dependent thylakoid protein translocase (Chaddock et al., 1995).

The thermolysin treatment of thylakoids from wild-type and mutant thylakoids analysed by immunoblot with specific antibodies (Figure 7A) shows that the mutant Cah3 is digested both in intact and sonicated thylakoids (lanes 4 and 6) while the wild-type protein is only degraded in the sonicated sample (lanes 3 and 5). The extrinsic oxygen-evolving enhancer protein OEE2, probed with a higher plant OEC-24 antibody, is used as a control (Figure 7B). The control lanes 7 and 8, where EDTA is added before thermolysin, show that no unspecific digestion is occurring.

**Impaired photosynthetic capacity of the CCM mutant, cia-3**

The mutant cia-3 has a severely impaired photosynthetic capacity under low C\textsubscript{i} conditions as determined by photosynthetic and chlorophyll a fluorescence measurements. Figure 8A shows the photosynthetic capacity as a function of the amount of bicarbonate added for high C\textsubscript{i} and 24 h low C\textsubscript{i}-adapted wild-type and mutant cells. The high C\textsubscript{i} cells were very similar with respect to their C\textsubscript{i} dependency. However, while the low C\textsubscript{i}-grown wild-type cells exhibited characteristically high C\textsubscript{i} affinity, the low C\textsubscript{i}-grown cia-3 cells had low affinity, similar to the affinity of cells grown in high C\textsubscript{i}. The maximum photosynthetic capacity of these cells was also lower, reaching only about half the photosynthetic rate of the other cell types. Figure 8B shows the C\textsubscript{i}-dependent response of the reduction of the primary electron acceptor (Q\textsubscript{A}), as estimated by 1 – F/Fm′ (Genty et al., 1989). This parameter is indicative of photosystem II (PSII) efficiency, and it clearly shows that the low C\textsubscript{i}-grown mutant cells suffer from a severe decrease in PSII efficiency compared with the wild-type. Even under higher bicarbonate concentrations, the mutant did not recover PSII efficiency or normal rates of photo-
synthesis. The reduction of photosynthetic capacity of the mutant after the transfer from high to low Ci conditions also increased with time (data not shown). Photosynthetic rates in the mutant dropped after 2–4 h at low Ci, and had decreased by 35–40% after 24 h.

Discussion

Cah3 is an α-type intracellular carbonic anhydrase in Chlamydomonas reinhardtii

We have described in this study the cloning and sequencing of a gene which encodes an intracellular CA of the α-type from C.reinhardtii. The deduced amino acid sequence of a full-length cDNA contained both the tryptic fragments and the N-terminus reported earlier (Karlsson et al., 1995). The predicted Cah3 polypeptide is the first α-type CA to be found within a photosynthetic eukaryotic cell. Although it has relatively low sequence similarity to animal α-type CAs (40% identity), the algal homologue has most of the conserved domains characteristic of this CA family. Most prominent are the three histidine residues that act as ligands for the Zn atom in the active site of the protein; residues 160, 162 and 179 in Cah3 (Figure 1A). The CA activity of the purified enzyme, measured by Karlsson et al. (1995), also confirms that this is a CA.

Chloroplastic localization of Cah3

Using specific antibodies raised against the 29.5 kDa CA protein, we have shown that Cah3 is located in the chloroplast, where it is localized exclusively in the thylakoid membrane, and is not associated with the chloroplast envelope (Figure 3). Previous mass spectrometric measurements on intact cells and isolated organelles led Amoroso et al. (1996) to suggest the presence of three intracellular CAs, two of which were believed to be associated with the chloroplast. One of the chloroplast forms was proposed to be a soluble CA while the other was believed to be membrane-associated. In our earlier work (Karlsson et al., 1995), however, we found evidence for only one intracellular, membrane-associated CA activity. In this study, using specific Cah3 antisera, we found no evidence for more than one α-type CA. This of course does not exclude the possibility that other types of CA (β or γ) may exist in the chloroplast of C.reinhardtii.

Sequence analyses of the Cah3 (Figure 1C) pre-sequence reveal striking similarities with pre-sequence known to specify translocation of proteins into the thylakoid lumen. The sequence also fulfils the criteria for transfer peptides translated via the ΔpH-dependent thylakoidal protein translocase; the characteristic twin arginine motif and a C-terminal lysine residue (Chaddock et al., 1995; Bögisch et al., 1997; reviewed by Robinson and Mant, 1997). The sequence information together with Western blot data (Figure 3) supports our belief that Cah3 is a protein located in the thylakoid lumen.

All immunological data were confirmed using antibodies raised against a Cah3 polypeptide produced in Escherichia coli by overexpression of the region of the cah3 gene encoding the mature Cah3 polypeptide (data not shown).

Expression of cah3 increases when the external CO₂ level decreases

There was an approximate doubling of cah3 mRNA in relation to the amount of rRNA present when cells were transferred from high to low Ci conditions (Figure 2). This level of induction is consistent with that proposed for the three intracellular CAs apparently induced under these conditions—which are estimated to increase 2- (Amoros et al., 1996) to 10-fold (Sültemeyer et al., 1995). A corresponding rise in CA protein content was not apparent on immunoblots, although it is possible that post-transcriptional and/or -translational regulation occurs to maintain a steady-state level of this CA in C.reinhardtii.

Inactive intracellular CA is present in the high Ci-requiring mutant, cia-3

Immunological examination of total cellular protein extract from the high Ci-requiring mutant, cia-3, indicated that it contained amounts of Cah3 similar to that of the wild-type (Figure 6). However, the CA-directed photoaffinity labelling showed that a 30 kDa polypeptide was labelled in wild-type cell extracts but not in the cia-3 mutant extracts. Although the obvious explanation was that cia-3 lacked this 30 kDa polypeptide, the CA identified by Husic and Marcus (1994) and the CA we have isolated previously (Karlsson et al., 1995) are very probably the same intracellular CA, since complementation of the mutant with the cah3 gene resulted in cell lines which produced the photoaffinity-labelled protein (Figure 5). The transformants also regained intracellular CA activity.

This hypothesis was strengthened further by crossing cia-3 with wild-type cells. Two of the resultant tetrad progeny (TD1 and TD3) showed mutant characteristics; a low efficiency for Ci utilization coupled with the absence of the 30 kDa labelled polypeptide (Table I), whereas the other two progeny (TD2 and TD4) showed both the presence of the 30 kDa photoaffinity-labelled polypeptide and the ability to grow on low CO₂, characteristic of wild-type cells. Since all progeny showed cross-reaction of a 30 kDa polypeptide with the Cah3-specific antibodies, it is therefore unlikely that cia-3 lacks this polypeptide.

We next considered whether the activity of the chloroplastic CA in cia-3 had been impaired by a mutation which caused a loss of the binding of the sulfonamide photoaffinity label inhibitor. Sulfonamides have been shown to bind to the active site cavity of an α-CA (Liljas et al., 1994). Unexpectedly, sequencing of the cah3 genomic DNA from cia-3 did not reveal a mutation in the mature part of the protein, but instead revealed two point mutations in the sequence encoding the transfer peptide (Figure 1C), leading to changes in the amino acid composition there; an arginine and methionine for two leucines. These changes occur one amino acid after the twin arginine motif, which is known to be important for the trans-thylakoidal proton gradient transport mechanism (Chaddock et al., 1995). Thus, the inactivation of the Cah3 protein is not caused by a mutation within the mature enzyme, but by sequence changes in the transfer peptide, which may lead to mistargeting, misfolding or incorrect cleavage of the Cah3 precursor. A mistargeting of the Cah3 polypeptide could in turn lead to improper folding and/or diminished activity because of the altered environment. In fact, the digestion of the mutant Cah3 in intact thylakoids (Figure 7) indicates that this protein is accessible for proteolytic attack while the wild-type protein is not. This strengthens both the assumption that the mutant has an impaired import of the Cah3 precursor into
the lumen and that the wild-type mature Cah3 is located extrinsically in the thylakoid lumen. The wild-type pattern equals that of the control protein, OEE2.

Expression of the cah3 gene in the cia-3 mutant was confirmed by Northern hybridization (data not shown). The fact that the mutant expresses full-length transcripts, although at low levels, supports the conclusion suggested by the sequence data that a mutation in the transfer peptide is responsible for the inactivation of the cia-3 CA.

Function of thylakoid lumen CA

After exposure to low C\textsubscript{i} conditions for 24 h, the high C\textsubscript{i}-requiring mutant, cia-3, suffers severely from low PSII efficiency and cannot sustain positive net photosynthesis during non-saturating light conditions, as shown by the results in Figure 8. It was reported earlier that this mutant accumulates C\textsubscript{i} inside the cell (Moroney et al., 1986) but still does not grow at ambient levels of CO\textsubscript{2}. Our discovery of a lumen-targeted CA is the experimental support for models inferring the presence of a CA within the lumen. Pronina and Semenenko (1990) and Pronina and Borodin (1993) have proposed a model in which a hypothetical membrane-associated CA in the lumen dehydrates HCO\textsubscript{3}\textsuperscript{–} taken up from the stroma in parallel with the active light-driven H\textsuperscript{+} influx. In the light, CO\textsubscript{2} is the major C\textsubscript{i} form at equilibrium in the acidic lumen, and this could then passively diffuse through the thylakoid membrane to a carboxylating site in the stroma close to the outer thylakoid membrane surface (Pronina and Semenenko, 1990) raising the effective CO\textsubscript{2} concentration available to Rubisco. This model with a lumenal CA has been developed further and analysed quantitatively by Raven (1997) and found to be qualitatively plausible. Our results from the mutant work support Raven’s model in that the mutant still accumulates C\textsubscript{i}, but in the high pH of the stroma in light, it occurs predominantly as HCO\textsubscript{3}\textsuperscript{–} and thus cannot act as substrate for Rubisco. The uncatalysed conversion of CO\textsubscript{2} to CO\textsubscript{2} in the stroma is also too slow to support high rates of photosynthesis. According to the model, HCO\textsubscript{3}\textsuperscript{–} is taken up into the lumen but in the mutant the catalysed conversion of HCO\textsubscript{3}\textsuperscript{–} to CO\textsubscript{2} does not take place, thus Rubisco cannot be supported fully with substrate via this pathway either. This would lead to a lowered CO\textsubscript{2} fixation rate, a lowered Calvin cycle turnover and, in turn, to a down-regulation of PSII efficiency (Baker and Boyer, 1994), as seen in Figure 8.

However, it has also been suggested that CA is associated with the photosynthetic electron transport chain close to PSI since certain inhibitors of CA also inhibit PSII activity (Stemler and Jursinic, 1983; Stemler, 1985). The mechanism for the ‘bicarbonate effect’ on PSI, discovered by Warburg and Krippahl (1960) and reviewed by Govindjee (1995), is still not elucidated fully. It has been proposed that a thylakoid CA might be involved in this bicarbonate effect (Moubarak-Milad and Stemler, 1994), ensuring that HCO\textsubscript{3}\textsuperscript{–} ions are available to stimulate effective transport of electrons through PSI. Although evidence for this proposal is so far preliminary, the identification of Cah3 as a thylakoid-associated CA allows us to test this hypothesis experimentally for the first time. We are now pursuing a more detailed examination of the specific role(s) of this CA and its specific location with respect to PSI and PSII centres in C.

Summary

In this report, we have shown by molecular complementation that the protein absent in strain cia-3 is an active Cah3. In addition, only transformants expressing an active Cah3 can grow at ambient levels of CO\textsubscript{2}. These results are consistent with the idea that the chloroplast thylakoid CA encoded by cah3 is an essential constituent of the enzymatic system that regulates photosynthetic carbon assimilation in C.

Materials and methods

Algal strains and culture conditions

Wild-type C.

Expression of the cDNA library, cloning and sequencing

A cDNA library was constructed using the Stratagene Uni-ZAPII kit (Stratagene, La Jolla, CA) from 5 μg of mRNA isolated from C.

Screening of cDNA library, cloning and sequencing

A cDNA library was constructed using the Stratagene Uni-ZAPII kit (Stratagene, La Jolla, CA) from 5 μg of mRNA isolated from C.

Identification of Cah3 as a thylakoid-associated CA allows us to test this hypothesis experimentally for the first time. We are now pursuing a more detailed examination of the specific role(s) of this CA and its specific location with respect to PSI and PSII centres in C.

Function of thylakoid lumen CA

After exposure to low C\textsubscript{i} conditions for 24 h, the high C\textsubscript{i}-requiring mutant, cia-3, suffers severely from low PSII efficiency and cannot sustain positive net photosynthesis during non-saturating light conditions, as shown by the results in Figure 8. It was reported earlier that this mutant accumulates C\textsubscript{i} inside the cell (Moroney et al., 1986) but still does not grow at ambient levels of CO\textsubscript{2}. Our discovery of a lumen-targeted CA is the experimental support for models inferring the presence of a CA within the lumen. Pronina and Semenenko (1990) and Pronina and Borodin (1993) have proposed a model in which a hypothetical membrane-associated CA in the lumen dehydrates HCO\textsubscript{3}\textsuperscript{–} taken up from the stroma in parallel with the active light-driven H\textsuperscript{+} influx. In the light, CO\textsubscript{2} is the major C\textsubscript{i} form at equilibrium in the acidic lumen, and this could then passively diffuse through the thylakoid membrane to a carboxylating site in the stroma close to the outer thylakoid membrane surface (Pronina and Semenenko, 1990) raising the effective CO\textsubscript{2} concentration available to Rubisco. This model with a lumenal CA has been developed further and analysed quantitatively by Raven (1997) and found to be qualitatively plausible. Our results from the mutant work support Raven’s model in that the mutant still accumulates C\textsubscript{i}, but in the high pH of the stroma in light, it occurs predominantly as HCO\textsubscript{3}\textsuperscript{–} and thus cannot act as substrate for Rubisco. The uncatalysed conversion of CO\textsubscript{2} to CO\textsubscript{2} in the stroma is also too slow to support high rates of photosynthesis. According to the model, HCO\textsubscript{3}\textsuperscript{–} is taken up into the lumen but in the mutant the catalysed conversion of HCO\textsubscript{3}\textsuperscript{–} to CO\textsubscript{2} does not take place, thus Rubisco cannot be supported fully with substrate via this pathway either. This would lead to a lowered CO\textsubscript{2} fixation rate, a lowered Calvin cycle turnover and, in turn, to a down-regulation of PSII efficiency (Baker and Boyer, 1994), as seen in Figure 8.

However, it has also been suggested that CA is associated with the photosynthetic electron transport chain close to PSI since certain inhibitors of CA also inhibit PSII activity (Stemler and Jursinic, 1983; Stemler, 1985). The mechanism for the ‘bicarbonate effect’ on PSI, discovered by Warburg and Krippahl (1960) and reviewed by Govindjee (1995), is still not elucidated fully. It has been proposed that a thylakoid CA might be involved in this bicarbonate effect (Moubarak-Milad and Stemler, 1994), ensuring that HCO\textsubscript{3}\textsuperscript{–} ions are available to stimulate effective transport of electrons through PSI. Although evidence for this proposal is so far preliminary, the identification of Cah3 as a thylakoid-associated CA allows us to test this hypothesis experimentally for the first time. We are now pursuing a more detailed examination of the specific role(s) of this CA and its specific location with respect to PSI and PSII centres in C.

Summary

In this report, we have shown by molecular complementation that the protein absent in strain cia-3 is an active Cah3. In addition, only transformants expressing an active Cah3 can grow at ambient levels of CO\textsubscript{2}. These results are consistent with the idea that the chloroplast thylakoid CA encoded by cah3 is an essential constituent of the enzymatic system that regulates photosynthetic carbon assimilation in C.

Materials and methods

Algal strains and culture conditions

Wild-type C.

Expression of the cDNA library, cloning and sequencing

A cDNA library was constructed using the Stratagene Uni-ZAPII kit (Stratagene, La Jolla, CA) from 5 μg of mRNA isolated from C.

Screening of cDNA library, cloning and sequencing

A cDNA library was constructed using the Stratagene Uni-ZAPII kit (Stratagene, La Jolla, CA) from 5 μg of mRNA isolated from C.

Identification of Cah3 as a thylakoid-associated CA allows us to test this hypothesis experimentally for the first time. We are now pursuing a more detailed examination of the specific role(s) of this CA and its specific location with respect to PSI and PSII centres in C.

Summary

In this report, we have shown by molecular complementation that the protein absent in strain cia-3 is an active Cah3. In addition, only transformants expressing an active Cah3 can grow at ambient levels of CO\textsubscript{2}. These results are consistent with the idea that the chloroplast thylakoid CA encoded by cah3 is an essential constituent of the enzymatic system that regulates photosynthetic carbon assimilation in C.
Genomic clones of the cah3 gene in the mutant cia-3 were obtained by PCR using cia-3 genomic DNA as template and primers designed using the wild-type cah3 gene. The resulting 1760 bp fragment, containing the complete coding strand, was cloned into pUC19 (Sambrook et al., 1989). Eight isolated plasmids were sequenced and confirmed as being identical.

**RNA blot hybridization**

Total nucleic acids were isolated from algae grown in high and low C i according to Johannesmeier and Howell (1984). RNA was purified using the RNeasy Total RNA Kit (Qagen). Northern blot analysis of RNA (10 µg) denatured by glyoxylation was performed according to Sambrook et al. (1989). Transcripts of the cah3 gene were detected using a 32 P-labelled cDNA which encodes the low C i-inducible mitochondrial CA (Eriksson et al., 1996) was used as a control for CCM induction. Hybridization and washing steps were done at 50°C according to Sambrook et al. (1989). The amount of specific mRNA in each sample was determined directly from the filter using a GS 250 Molecular Imager (Bio-Rad) and accompanying PhosphoImager software, and quantified relative to the amount of 16S/18S rRNA as described in Kulharni et al. (1992).

**Protein electrophoresis, cell fractionation, immunoblotting and photoaffinity labelling**

For protein analyses, cells were harvested and washed once in a buffer containing 25 mM HEPES-KOH pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride and 0.1 mM dithiothreitol (DTT). Samples were resuspended in a buffer containing 20 mM bis-tris-propane pH 7.0, 5 mM MgCl2, 5 mM DTT, 0.2 mM ATP, 10 µM leupeptin and 2 mM benzamidine, then frozen at –80°C until used for electrophoresis. Intact chloroplasts were isolated as described by Mason et al. (1991), and chloroplast envelope membranes as described by Ramazanov et al. (1993). Thylakoid membranes were isolated according to the method of Allen and Staehelin (1993). Proteins were separated on 8–15% gradient polyacrylamide gels (0.8% bisacrylamide) as described previously (Laemmli, 1970). Immunoblotting was performed as described in the protocol from Bio-Rad Laboratories. Antibodies directed against a synthetic peptide derived from an internal amino acid sequence of Cah3 (residues 213–224; Figure 5A) were obtained from Research Genetics (Huntsville, AL).

Chlamydomonas reinhardtii cell extracts for photoaffinity labelling experiments were prepared and were labelled with the CA-directed photoaffinity reagent [125 I]PAMBS-ASA, as described previously (Husic and Marcus, 1994). Thermolysin treatments of isolated Cah3 wild-type (cw15) and Cah3 mutant (cia/cw15) thylakoids were done as described previously (Brock et al., 1993). In the reactions, 75 µg/ml thermolysin was used for chlorophyll concentrations of 250 µg/ml. After the incubation, the reactions were stopped with the addition of EDTA. The samples were analysed after denaturing gel electrophoresis and immunoblotting as described above. The antibodies used were Cah3 and OEE2 specific (raised against pea OEC-24, originally from Dr R.Henry, University of Florida).

**Photosynthesis and chlorophyll fluorescence parameters**

Photosynthetic O2 exchange rates at 400 µmol photons/m²/s of white light were measured at 25°C using a Clark-type O2 electrode (Hansatech, King’s Lynn, UK) with cells suspended to 3 µg chlorophyll/ml in CO2-free growth media. The reaction was initiated by adding various concentrations of NaHCO3. During measurements of O2 evolution, the chlorophyll fluorescence parameters, F (chlorophyll fluorescence yield during illumination) and Fm’ (chlorophyll fluorescence yield when all PSII are closed transiently by a saturating light pulse of 500 ms duration and 6000 µmol photons/m²/s intensity) were measured simultaneously using a PAM chlorophyll fluorometer (Waltz, Effeltrich, Germany), and the efficiency of reduction of primary quinone electron acceptor (Qo) was estimated as (1 – F/Fm’). Genty et al., 1989).

**Acknowledgements**

We are very grateful to Dr S.Purton (University College London) for supplying the genomic library. This work was supported by the Swedish Natural Science Research Council (grants to G.S. and A.K.C.) and the National Science Foundation (grant No. DBM-9418502 to H.D.H. and grant No. IBN-9304662 to J.V.M.)

**References**


Pronina, N.A. and Borodin, V.V. (1993) CO₂ stress and CO₂ concentration

Received July 2, 1997; revised November 17, 1997; accepted December 29, 1997.