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Carbonic anhydrase and carbonic anhydrase like genes of Chlamydomonas reinhardtii

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CARBONIC ANHYDRASE AND CARBONIC ANHYDRASE
LIKE GENES OF CHLAMYDOMONAS REINHARDTII

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

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I am indebted to all my friends and well wishers who have tolerated my eccentricities for all these years and have never abandoned me. Finally, I would like to thank my parents for their encouragement and continuous support and above all, their unconditional love. Without them, this document would never have been completed.
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<th>Description</th>
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AZ</td>
<td>Acetazolamide</td>
</tr>
<tr>
<td>BBY</td>
<td>Berthold-Babcock-Yocum PSII particles</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>CAs</td>
<td>Carbonic anhydrases</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean acid metabolism</td>
</tr>
<tr>
<td>CAP</td>
<td>Contig assembly program</td>
</tr>
<tr>
<td>Ci</td>
<td>Inorganic carbon</td>
</tr>
<tr>
<td>CCM</td>
<td>CO$_2$ concentrating mechanism</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytosine triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>Mixture of dATP, dCTP, dGTP and dTTP</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5′, 5′-dithiobis(2-nitrobenzoic azide)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EZ</td>
<td>Ethooxyzolamide</td>
</tr>
<tr>
<td>EPPS</td>
<td>4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GAP</td>
<td>Glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>Gclp</td>
<td>Gamma CA like protein</td>
</tr>
<tr>
<td>HCR</td>
<td>High CO$_2$ requiring phenotype</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β, D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Mer</td>
<td>2- Mercaptoethanol</td>
</tr>
<tr>
<td>MIN</td>
<td>Minimal media</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige-Skoog</td>
</tr>
<tr>
<td>MOPS</td>
<td>Morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>β- nicotinamide adenine dinucleotide, oxidized form</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>β- nicotinamide adenine dinucleotide phosphate, oxidized form</td>
</tr>
<tr>
<td>NADPH</td>
<td>β- nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate saline buffer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>3-PGA</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PS</td>
<td>Photosystem</td>
</tr>
<tr>
<td>PSR</td>
<td>Proton shuttle residue</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rbcL</td>
<td>Rubisco large subunit</td>
</tr>
</tbody>
</table>
Rubisco  Ribulose 1, 5 bisphosphate carboxylase /oxygenase
RuBP    Ribulose 1, 5 bisphosphate
SDS     Sodium dodecyl sulfate
TAP     Tris Acetate Phosphate
WAU     Wilbur and Anderson unit
WT      Wild type
X-Gal   5-bromo-4-chloro-3-indolyl-β-D-galactoside
ABSTRACT

Carbonic anhydrase (CA) is a zinc containing metalloenzyme that catalyzes the reversible interconversion of $\text{CO}_2$ and $\text{HCO}_3^-$. There are three evolutionarily unrelated CA families designated $\alpha$-, $\beta$-, and $\gamma$-CA. Vertebrates have members of the $\alpha$-CA family, while higher plants, algae and cyanobacteria have members belonging to all three CA families. In the green alga, *Chlamydomonas reinhardtii*, five CAs have previously been identified including three $\alpha$-CAs and two $\beta$-CAs. This dissertation describes the identification and characterization of new CA genes from *C. reinhardtii*. Four new CA or CA-like genes have been discovered including two $\beta$-CAs and two $\gamma$-CAs. Three CAs were investigated further including the $\alpha$-CA Cah3, one of the new $\beta$-CAs, Cah6; and a new $\gamma$-CA designated Gclp1 for gamma-CA-like protein.

Cah3 is an $\alpha$-CA located in the thylakoid. Past studies with two Cah3 mutants, *ca-1* and *cia3* have shown that Cah3 plays an important role in the $\text{CO}_2$ concentrating mechanism. In this work, the mature Cah3 protein was overexpressed as a fusion protein in *E. coli* and found to have significant CA activity. This is the first report of detection of CA activity in the Cah3 protein and its partial biochemical characterization.

A novel $\beta$-CA (*Cah6*) and a putative $\gamma$-CA (*Gclp1*) gene were identified in *C. reinhardtii*. Gclp1 is one of two putative $\gamma$-CAs found in *C. reinhardtii*. Both the Cah6 and Gclp1 open reading frames (ORFs) were cloned in the overexpression vector pMal-c2x and expressed as recombinant fusion proteins. The purified Cah6 had significant *in vitro* CA activity but Gclp1 did not. Gclp1 was designated as a $\gamma$-CA like protein because it lacked detectable CA activity. Cah6 has a leader sequence consistent with a chloroplast localization. Although Cah6 is constitutively expressed under low and high $\text{CO}_2$ conditions, it is slightly upregulated under low $\text{CO}_2$ conditions. Immunolocalization studies confirmed that the Cah6 is localized to the
chloroplast stroma particularly in the starch sheath around the pyrenoid. A possible role of Cah6 in the CO₂ concentrating mechanism and photosynthesis is discussed.
CARBONIC ANHYDRASE - AN OVERVIEW

Carbonic anhydrase (carbonate dehydratase, carbonate hydrolyase; EC 4.2.1.1) is a zinc containing metalloenzyme that catalyzes the reversible interconversion of CO$_2$ and HCO$_3^-$ and has a maximum turnover number in excess of $10^5$ s$^{-1}$ (Khalifah, 1971). The enzyme was first discovered in human erythrocytes (Meldrum and Roughton, 1933) but has since been found in many organisms including animals, plants, archaebacteria and eubacteria (Hewett-Emmett and Tashian, 1996; Table 1.1). Carbonic anhydrase (CA) plays an important role in many physiological functions that involve decarboxylation or carboxylation reactions, including both photosynthesis and respiration. It also participates in the transport of inorganic carbon (C$_i$) to actively photosynthesizing cells or away from actively respiring cells (Henry, 1996).

The known CAs can be grouped broadly into three independent families (Hewett-Emmett and Tashian, 1996), α-CA, β-CA and γ-CA. These three families have no significant sequence identities, seem to have evolved independently and are an example of convergent evolution of catalytic function (Hewett-Emmett and Tashian, 1996). Although the primary sequences of these CA families are different, the active sites of these three types of CAs contain Zn$^{2+}$ and all of them employ a two-step catalytic mechanism (Lindskog, 1997).

The first step is a nucleophilic attack of a zinc-bound hydroxyl ion on a CO$_2$ molecule residing in a hydrophobic pocket, generating a zinc-bound HCO$_3^-$ (Equation 1). "E" represents the enzyme. The bicarbonate bound to the zinc is replaced by a water molecule, releasing HCO$_3^-$ (Equation 2). The HCO$_3^-$ can gain a H$^+$ to form H$_2$CO$_3$ or can lose an additional H$^+$ to form CO$_3^{2-}$ (Equation 3). The second step is the regeneration of the active site by the ionization of the
### Table 1.1 A summary of the different families of carbonic anhydrases

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Distribution</th>
<th>Inhibition</th>
<th>Zinc coordination</th>
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</thead>
<tbody>
<tr>
<td>α</td>
<td>vertebrates, algae, eubacteria, plants and viruses</td>
<td>highly susceptible to sulfonamides</td>
<td>three histidine residues **</td>
</tr>
<tr>
<td>β</td>
<td>algae, plants, eubacteria, fungi, invertebrates and archaeabacteria</td>
<td>less susceptible to sulfonamides</td>
<td>two cysteine residues and one histidine residue</td>
</tr>
<tr>
<td>γ</td>
<td>archaeabacteria, cyanobacteria, algae* and plants*</td>
<td>highly susceptible to sulfonamides</td>
<td>three histidine residues ***</td>
</tr>
<tr>
<td>δ</td>
<td>Thalassiosira weissflogii (diatom)+</td>
<td>not known</td>
<td>Sequence does not match a known α-, β- or a γ-CA</td>
</tr>
</tbody>
</table>

* putative genes exist in EST database ; ** histidine residues are contributed by the same subunit, *** histidine residues are contributed by two different subunits, + CA activity has not been found in the purified protein
water molecule bound to the zinc ion and removal of a proton from the active site. Most carbonic anhydrases have $k_{cat}$ values greater than $10^4 \text{ s}^{-1}$ which requires an intermediate proton shuttle residue (PSR) (Equation 4) to transfer the proton from the metal bound water molecule to the external buffer, “B” (Equation 5). In this step, the zinc ion acts as a Lewis acid to lower the pK$_a$ of the water molecule from ~ 14 to 7.0.

\[
\text{E-Zn}^{2+}-\text{OH}^- + \text{CO}_2 \leftrightarrow \text{E-Zn}^{2+}-\text{HCO}_3^- \text{ (Equation 1)}
\]

\[
\text{E-Zn}^{2+}-\text{HCO}_3^- + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{E-Zn}^2-\text{H}_2\text{O} \text{ (Equation 2)}
\]

\[
\text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow 2\text{H}^+ + \text{CO}_3^- \text{ (Equation 3)}
\]

\[
\text{PSR} + \text{E-Zn}^{2+}-\text{H}_2\text{O} \leftrightarrow \text{E-Zn}^{2+}-\text{OH}^- + \text{PSR-H}^+ \text{ (Equation 4)}
\]

\[
\text{PSR-H}^+ + \text{B} \leftrightarrow \text{PSR} + \text{B-H}^+ \text{ (Equation 5)}
\]

Proton transport from the active site is the rate limiting step for enzymes with $k_{cat} > 10^4 \text{ s}^{-1}$. Thus, the $k_{cat}$ is a reflection of the rate of proton transport (Equation 4 and 5), whereas the catalytic efficiency ($k_{cat}/K_m$) is more reflective of the hydration step (Equation 1) and is insensitive to the rate of proton transport. The overall relationship between the three forms of dissolved inorganic carbon ($C_i$) is shown in (Equation 6).

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{CO}_3^{2-} + 2\text{H}^+ \text{ (Equation 6)}
\]

The uncatalyzed hydration and dehydration reactions are slow while the dissociation reactions are considered instantaneous. CA accelerates the hydration of dissolved CO$_2$ in solution and increases the rate at which the different forms of $C_i$ interconvert in solution. The equilibrium between the $C_i$ is pH dependent. At physiological ionic strengths, if the pH level is below the first dissociation constant (pK$_1 \approx 6.4$), CO$_2$ predominates; at pH between 6.4 and at about 10.3 (pK$_2$); HCO$_3^-$ predominates; whereas at pH above 10.3, CO$_3^{2-}$ predominates.

In the first part of this chapter, the different types of CAs currently known, their structures and intracellular locations in photosynthetic organisms are discussed. In the second
part, the physiological roles of these CAs in photosynthesis and the CO₂ concentrating mechanisms in plants, cyanobacteria and algae have been emphasized along with a brief discussion of photosynthesis, the different CCMs and components of CCMs in plants, cyanobacteria and algae. This dissertation mainly focuses on the different CAs in the green alga *Chlamydomonas reinhardtii*.

1. α-Carbonic Anhydrases

The α-type is the most studied CA and is very widely distributed. α-CAs have been found in animals, plants, algae, eubacteria and viruses. α-CA was first identified in human red blood cells. Vertebrate α-CAs can be classified into two groups (Jiang and Gupta, 1999). One group includes the soluble isoforms like CAI, CAII, CAIII, CAIV, CAIX, CAXII and CAXIV. The second group includes membrane associated CAs like (CAIV, CAVI, CAIX, CAXII, CAXIV and CAXVI). Humans have eleven active isozymes including cytoplasmic (CAI, CAII, CAIII and CAVII), mitochondrial (CAVA and CAVB), secreted (CAVI) and membrane associated (CAIV, CAIX, CAXII and CAXIV) forms. Humans also have numerous carbonic anhydrase-related proteins (CA-RPs) that appear not to have CA activity (Sly and Hu, 1995). The range of the specific activities of these isozymes is quite large, with CAII having the highest [2000-3000 WAU/mg; (Wilbur and Anderson unit/mg)] and CAIII (1-5 WAU/mg) the lowest specific activity. All enzymatically active α-CAs have three histidine residues coordinating to the zinc atom (Fig. 1.1). These histidines as well as a number of other residues are well conserved in all active α-CAs. The α-CA structure is dominated by antiparallel β-sheet forming a spherical molecule with two halves. The active site is a funnel-shaped crater with the zinc atom located near the bottom.

In photosynthetic organisms only a few α-CAs have been identified at this time. Most α-CAs are active as monomers of about 30 kDa. One has been identified in each of the
cyanobacteria *Anabaena* and *Synechococcus* (Soltes-Rak *et al.*, 1997), and in both cases the CA is localized to the periplasmic space. In contrast, *Synechocystis* PCC6803 does not have an α-CA. The green alga *C. reinhardtii* (Table 1.2) (Karlsson *et al.*, 1998) and *Dunaliella salina* (Fisher *et al.*, 1996; Yang *et al.*, 1999) have three and two α-CAs, respectively. CAs from *D. salina* are located in the periplasm. One of the CA genes of *D. salina* encodes a protein of about 63 kDa that appears to have two active sites, possibly the results of gene duplication and fusion events (Fisher *et al.*, 1996).

In *C. reinhardtii*, two of the α-CAs are located in the periplasm and one in the thylakoid lumen. The first α-CA genes cloned from a photosynthetic organism were *Cah1* and *Cah2*, which encode the two periplasmic CAs in *C. reinhardtii* (Fukuzawa *et al.*, 1990; Fujiwara *et al.*, 1990). These two genes encode very similar proteins although they are differently regulated. *Cah1* is expressed under low CO₂ conditions but not under high CO₂ conditions. In contrast, *Cah2* is poorly expressed under low CO₂ and slightly upregulated under high CO₂ conditions. In addition, the expression of *Cah2* under high CO₂ appears low compared to the expression of *Cah1* under low CO₂ (Fujiwara *et al.*, 1990; Rawat and Moroney, 1991). Possibly, *Cah2* resulted from a gene duplication event and has a poorly functioning promoter. *Cah1* holoenzyme is a heterotetramer with two 37 kDa subunits and two 4 kDa subunits held together by disulfide bonds (Kamo *et al.*, 1990).

The third α-CA gene in *C. reinhardtii*, *Cah3*, was discovered in 1995 (Karlsson *et al.*, 1995). The deduced full length protein contained an amino terminal extension characteristic of bipartite leader sequence. This information along with the immunolocalization studies indicate that this carbonic anhydrase is localized in the lumen of the thylakoid membrane. This α-CA is constitutively expressed and is slightly upregulated under low CO₂ conditions (Karlsson *et al.*, 1998). *Cah3* is a 29 kDa hydrophobic protein with a pI of ~7.87. It is not a membrane spanning
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<tr>
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</tr>
<tr>
<td>HumanCAI</td>
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</tr>
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<td>Synechococcus</td>
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</table>

**Figure 1.1 Multiple sequence alignment of α-CAs.** The *C. reinhardtii* CA sequence shown above represents that of thylakoid α-CA, Cah3. Active site residues are shown in bold red. * represents a completely conserved amino acid, : represent conserved amino acid substitutions, and . represent semi conserved amino acid substitutions.
<table>
<thead>
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<th>Known gene/s</th>
<th>Location</th>
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<td>Periplasm</td>
<td>Fukuzawa et al. 1990; Fujiwara et al. 1990</td>
</tr>
<tr>
<td>α</td>
<td>Cah3</td>
<td>Thylakoid</td>
<td>Karlsson et al. 1995</td>
</tr>
<tr>
<td>β</td>
<td>Ca1, Ca2</td>
<td>Mitochondria</td>
<td>Eriksson et al. 1996</td>
</tr>
</tbody>
</table>
protein as it can be released into the soluble fraction by washing the thylakoids with 300 mM KCl (Karlsson et al. 1998). Although the Cah3 gene has been sequenced, there is no report of the detection and measurement of CA activity of the purified protein. Recently, CA activity of purified recombinant Cah3 was measured and partial biochemical characterization of the purified enzyme was done (Chapter 3 of this dissertation).

It is clear that a number of α-CAs remain to be identified in higher plants. Currently the Arabidopsis thaliana database contains six different genes that align with α-CAs (Moroney et al., 2001). To our knowledge, only one α-CA cDNA from a higher plant, ACAHI from A. thaliana, has been completely sequenced (Moroney et al., 2001). However a few ESTs in the Arabidopsis database do align with some of the other α-CAs identified by the genome project, suggesting that these are expressed genes.

A number of nongreen plant tissues also appear to have CA activity based on the observed high rates of HCO\textsubscript{3} consumption (Raven and Newman, 1994). Coba de la Pena et al. (1997) showed that an α-CA is present in both spontaneously formed and in Rhizobium meliloti-induced root nodules of alfalfa. This α-CA is expressed early in the nodule primordium and found later in both developing and mature nodules.

2. β-Carbonic Anhydrases

The β-CAs do not appear to be as broadly distributed as the α-CAs at this time. β-CAs were first recognized to be CAs in photosynthetic organisms (Fawcett et al., 1990; Burnell et al., 1990). Once recognized as a CA, β-CAs have been found in higher plants, algae, eubacteria (Hewett-Emmett and Tashian, 1996), archaeabacteria (Smith and Ferry, 1999), the fungi Saccharomyces cerevisiae and S. pombe (Götz et al., 1999), Caenorhabditis elegans and Drosophila melanogaster, but not in vertebrates. In C\textsubscript{3} plants, the abundant β-CA is localized to the chloroplast stroma and is a highly active CA. β-CAs have been found both in the cytoplasm
(C₄ mesophyll cells) and chloroplast (C₃ plants) in higher plants and in the cytoplasm of the symbiotic alga *Coccomyxa* (Hiltonen *et al*., 1998).

X-ray absorption spectroscopy studies on spinach β-CAs have shown that a histidine and two cysteine residues are the zinc coordinating residues (Bracey *et al*., 1994; Rowlett *et al*., 1994) (Fig. 1.2). Recently the crystal structures of a β-CA from *Porphyridium purpureum* (Mitsuhashi *et al*., 2000) and *Pisum sativum* (Kimber and Pai, 2000) were resolved. The pea CA is an octamer in which dimers form tetramers which form octamers. The red algal CA is double the size of the spinach CA and has two active sites per polypeptide instead of the one found in other β-CAs from algae and higher plants, indicating that a gene duplication event occurred (Mitsuhashi and Miyachi, 1996; Mitsuhashi *et al*., 2000). In the algal CA, an aspartate rather than water occupies the fourth coordination position (Mitsuhashi *et al*., 2000). The red algal CA was crystallized in the absence of substrate (or inhibitor) while the pea CA was crystallized in the presence of acetate. Most probably the difference between the zinc ligands of the two enzymes is due to the conditions under which the crystals were formed. The notion that the CO₂ residing in a hydrophobic pocket is required for activity is underscored by the recent comparison between the α-CA and β-CA crystal structures (Kimber and Pai, 2000). It has been found that the three dimensional structure of the active site of the β-CA is a mirror image of that of the active site of the α-CA (Kimber and Pai, 2000).

β-CAs have been found in all photosynthetic organisms studied to date. In the cyanobacterium *Synechocystis* PCC 6803, a β-CA has been described which is encoded by the *icfA* gene. This CA appears to be localized to the carboxysome (Fukuzawa *et al*., 1992; Yu *et al*., 1992). Loss of the carboxysomal β-CA leads to high CO₂ requiring phenotype (HCR) (Price and Badger, 1989b). This is the only β-CA in this bacterium as the genome contains no other β-CA gene.
**Synechococcus**

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**Coccomyxa**

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**Spinach**

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**Synechococcus**

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**Coccomyxa**

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**Spinach**

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**Synechococcus**

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**Spinach**

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Figure 1.2  Multiple sequence alignment of β-CAs. The full length CAs of *Synechococcus*, *Coccomyxa* and spinach are 272, 227 and 319 amino acids long, respectively. In the figure, 26 amino acids at the carboxyl end of *Synechococcus* CA and 60 amino acids at the amino terminal end of spinach CA are not shown. Active site residues are shown in bold red. * represents a completely conserved amino acid, : represent conserved amino acid substitutions, and . represent semi conserved amino acid substitutions.
Two nearly identical β-CAs, were identified in 1996 in *C. reinhardtii* (Table 1.2) (Eriksson *et al*., 1996). Both the β-CAs are located in the mitochondria. The molecular weight of the mitochondrial β-CA protein is approximately 21 kDa. These CAs are expressed only under low CO$_2$ conditions (0.035%) and are absent under high CO$_2$ (5%) conditions (Eriksson *et al*., 1998). Recently Giordano *et al*., (2003) have shown that the expressions of these CAs are induced at 0.2% CO$_2$ conditions by increasing the NH$_4^+$ concentration in the growth medium. These workers have proposed that the mitochondrial CAs are involved in providing carbon flux in the form of HCO$_3^-$ to the Tri Carboxylic Acid cycle (TCA) cycle via anaplerosis. The discovery of a third β-CA (*Cah6*) gene in *C. reinhardtii* is reported in Chapter 4.

In *Arabidopsis thaliana*, cDNAs encoding the cytoplasmic and chloroplastic forms of β-CAs have been described (Fett and Coleman, 1994). At this time sequences encoding at least five β-CA genes from *Arabidopsis* are in the database and it is possible that other β-CAs might be found in the future. The C$_4$ monocot plants *Zea mays* (Burnell *et al*., 1999) and *Urochloa panicoides* (Ludwig and Burnell, 1995) also have β-CAs. Translation of the two CA cDNAs in the maize database yields significantly larger CA proteins of about 74 and 60 kDa (Burnell *et al*., 1999). These large polypeptides appear to be a fusion of two monomers since they contain two sets of active site residues. The quaternary structures of the maize CAs have not yet been deciphered. The unusual size of the maize CA protein is unique among higher plants since the cDNAs of C$_4$ plants *U. panicoides* and *Flaveria bidentis* appear “normal” in size and have molecular weights between 24 and 30 kDa. However the fused gene seen in maize is reminiscent of the *Porphyridium* CA (Mitsuhashi *et al*., 2000). Interestingly, all monocot CAs for which sequence information is available lack twelve amino acid residues at the carboxyl terminal end, which are conserved in the dicot CAs and play a role in the oligomerization of the pea CA (Kimber and Pai, 2000).
3. $\gamma$-Carbonic Anhydrases

A third type of CA, the $\gamma$-CA, was discovered in the archaebacterium *Methanosarcina thermophila* (Alber and Ferry, 1994). Genes encoding putative $\gamma$-CA proteins have been found in eubacteria and plants (Newman *et al*., 1994). The $\gamma$-CA from *M. thermophila* was crystallized and its structure solved (Kisker *et al*., 1996). The structure of the $\gamma$-CA is remarkably different from that of $\alpha$-CA or $\beta$-CA. The $\gamma$-CA functions as a trimer of identical subunits. Each monomer is a left-handed $\beta$-helix (Kisker *et al*., 1996). The trimer contains three zinc atoms, each at the three subunit interfaces. As in $\alpha$-CAs, three histidines and a water molecule coordinate the zinc atom but the histidines are provided by two subunits (Fig. 1.3). For the *M. thermophila* protein, His 81 and His 122 from one subunit act as ligands and His 117 from a different subunit is the third ligand. In spite of the fact that the active site is at the subunit interface, architecturally the active site of $\gamma$-CA resembles that of $\alpha$-CA (Kisker *et al*., 1996).

A $\gamma$-CA homologue, *CcmM* was discovered earlier in *Synechococcus* PCC7942 (Price *et al*., 1993). *CcmM* lacks detectable CA activity but it is required for growth under low CO$_2$ conditions (Moroney *et al*., 2001). If this gene is mutated, the mutant cannot grow on air levels of CO$_2$, suggesting that it is an essential part of CCM (Price *et al*., 1993). The *CcmM* protein is over 300 amino acids longer than the *M. thermophila* protein. The N-terminal portion of *CcmM* has a high homology to the archebacterial $\gamma$-CA. The C-terminal portion of *CcmM* has three to four 87 amino acid repeats that are very similar to the small subunit of Rubisco from the cyanobacteria (Price *et al*., 1998). The exact role of *CcmM* in the CCM is not clear. The *C. reinhardtii* EST database also has two ESTs that align well with the *M. thermophila* $\gamma$-CA. Genomic sequences were obtained for both these gamma CA-like protein genes (*Gclp1* and *Gclp2*). One of them, *Gclp1* has been cloned and overexpressed in *Escherichia coli*. A detailed discussion about *Gclp1* is in chapter 5 of this dissertation. Several *Arabidopsis* ESTs in the
databases have homology to the \( \gamma \)-CA from *M. thermophila* (Moroney *et al*., 2001). When the databases are searched using sequences around the putative active site of one of these ESTs, three different genomic sequences are obtained. Two of them, one from chromosome I and the other from chromosome V, have very similar sequences around the active site. The third one, a shotgun clone, is also located on chromosome 1 and is much less similar but retains the histidines at the active site (Moroney *et al*., 2001).

4. **Unresolved Questions: Are There More CA Gene Families?**

In 1997, a cDNA encoding a CA from the diatom *Thalassiosira weissflogii* was described by Roberts *et al*. The CA was isolated and a partial amino acid sequence was obtained from the purified protein. Using this protein sequence information, primers were designed to screen a *T. weissflogii* cDNA library by polymerase chain reaction (PCR). The amplified putative CA cDNA sequence was cloned and sequenced. The protein sequence of this CA does not match any known CA sequences. The authors have named the CA family as \( \delta \)-CA. This raises the possibility that there might be another CA gene family.

Unfortunately the authors could not overexpress the protein in *E. coli* and obtain CA activity. Furthermore, there have been no further reports of CA cDNAs that match the cDNA obtained from *Thalassiosira weissflogii*. Future work is needed to ascertain that there is indeed a \( \delta \)-CA family, in addition to the other three gene families. Morel’s group raised an additional question whether or not the cadmium can substitute for zinc in CA, or if there is a separate cadmium-dependent CA (Lane and Morel, 2000).

Recent work with *Thalassiosira weissflogii* indicates that this diatom might be able to substitute cadmium or cobalt for zinc under zinc limiting environment (Lane and Morel, 2000). The workers demonstrated that the cadmium-or cobalt-CA is different from the CA that they had previously identified in *T. weissflogii* (Roberts *et al*., 1997). This raises the possibility that there
Figure 1.3  Multiple sequence alignment of γ-CAs. Cam represents the γ-CA from *Methanosarcina thermophila* and CcmM represents the γ-CA from *Synechococcus* sp. Active site residues are shown in bold red. The γ-CA from *Methanosarcina* has an open reading frame of 247 amino acids. The CcmM protein from *Synechococcus* has a 290 amino acids long C-terminal extension. The complete CcmM protein from *Synechococcus* has an open reading frame of 539 amino acids. * represents a completely conserved amino acid, : represent conserved amino acid substitutions, and . represent semi conserved amino acid substitutions.
can be Cd or Co-requiring CAs in diatoms and also perhaps in algae and higher plants. It has been reported that Co can also substitute for Zn in the γ-CA from M. thermophila (Alber et al., 1999).

**RIBULOSE 1, 5 BISPHOSPHATE CARBOXYLASE /OXYGENASE (RUBISCO) - AN OVERVIEW**

Photosynthesis is the biological process by which most photoautotrophs reduce CO₂ to carbohydrates with the release of O₂ as a byproduct of the photochemical oxidation of water. The main enzyme participating in CO₂ fixation process is ribulose 1, 5 bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39). In the carboxylation reaction, Rubisco catalyzes the addition of CO₂ to ribulose 1, 5 bisphosphate (RuBP), generating two molecules of 3-phosphoglycerate (3-PGA). Molecules of 3-PGA are converted to glyceraldehyde-3-phosphate (GAP) via the Calvin cycle.

Rubisco can also utilize O₂ as a substrate. It catalyzes the oxygenation of ribulose 1, 5 bisphosphate, to produce one molecule of 3-PGA and one molecule of 2-phosphoglycolate, a compound that cannot be utilized in the C₃ reductive photosynthetic carbon cycle. A second cycle termed the C₂ oxidative photosynthetic carbon cycle (photorespiratory carbon oxidation cycle) salvages 75% of the constituent carbon utilizing ATP and NADPH (Hartman and Harpel, 1994). It seems that the oxygenase activity is intrinsic to Rubisco and reflects that the ancestral Rubisco evolved in an anaerobic atmosphere. Thus the relative rates of oxygenation and carboxylation by Rubisco are major factors in determining the efficiency of photosynthesis. Rubisco is catalytically a very slow enzyme with a turnover rate of 3-4 s⁻¹ compared to 10⁴ or 10⁵ s⁻¹ times for some enzymes like CA and 3-Keto steroid isomerase. The relative rates of catalysis of oxygenation or carboxylation by Rubisco is controlled by the relative concentrations of O₂ and CO₂ at the site where the enzyme is localized. The chloroplast stroma of mesophyll cells of
higher plants contain about 9 µM CO$_2$ and about 250 µM O$_2$ (Keys, 1986) at 25°C. The K$_{m}$ values of Rubisco for CO$_2$ and O$_2$ are approximately 8-25 µM and 360-650 µM (Jordan and Ogren, 1983; Keys 1986). As the concentration of CO$_2$ in the stroma of mesophyll cells is approximately equal to the K$_{m}$ for CO$_2$, Rubisco normally functions at only half of its already-low maximum velocity.

The oxygenation reaction competes with the carboxylation reaction, which slows this carboxylation rate by another 28% under current atmospheric conditions. Given a specificity factor of 100 for Rubisco, the ratio of carboxylase to oxygenase activity is approximately 3 to 1 under present atmospheric conditions. Thus to sustain reasonable rates of photosynthetic CO$_2$ fixation, plants must make an enormous amount of Rubisco. The enzyme may constitute up to 25% of the total nitrogen in a plant and up to 50% of the protein in the chloroplast stroma. Considering the quantity of photosynthetic tissue, Rubisco is probably the most abundant enzyme in the biosphere (Goodwin and Mercer, 1983).

In higher plants, cyanobacteria and most eukaryotic algae, Rubisco is composed of eight large subunits and eight small subunits. The holoenzyme has eight active sites. Each active site of Rubisco is catalytically active only after the formation of a ternary complex with CO$_2$ and Mg$^{2+}$. This reaction, called activation, begins with the covalent binding of a CO$_2$ molecule to the ε-amino group of a lysine residue in the large subunit. The resulting carbamate is then stabilized by the binding of Mg$^{2+}$ (Schneider et al., 1992). The CO$_2$ molecule that forms the carbamate is termed the activator CO$_2$ and is distinct form the substrate CO$_2$ molecule. In addition to stabilizing the carbamate, the metal ion plays an important role in some aspect of catalysis and may interact with the gaseous substrate. This is based on the finding that other divalent metal ions like Ni$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$ and Cu$^{2+}$ can substitute for Mg$^{2+}$ in stabilizing carbamate and the catalytic rates for these forms of the enzyme are low and differ in their ability to discriminate
between CO$_2$ and O$_2$ (Andrews and Lorimer, 1987). Mg$^{2+}$ binding may promote the proper binding of the phosphate groups of RuBP.

The reaction mechanism is strictly ordered. RuBP binds first and then reacts with either CO$_2$ or O$_2$. After binding of RuBP to an activated site, the first step in catalysis is the deprotonation of C3 to form a 2, 3-enediol, which creates a nucleophilic center at C2. At this point CO$_2$ and O$_2$ compete for the nucleophilic center at C2, with carboxylation producing two molecules of 3PGA and oxygenation producing one molecule of 3 PGA and one molecule of 2-phosphoglycolate. Because of the catalytic properties of Rubisco, CO$_2$ and O$_2$ are competitive substrates and an increase in the CO$_2$ concentration favors the carboxylation rate, leading to the effective fixation of CO$_2$ by Calvin cycle.

**FUNCTIONAL ROLES OF DIFFERENT CARBONIC ANHYDRASES IN THE PHOTOSYNTHETIC CARBON DIOXIDE CONCENTRATING MECHANISM (CCM)**

Higher plants, aquatic angiosperms, cyanobacteria and algae have all developed their own unique versions of photosynthetic CCMs to aid Ribulose 1, 5 bisphosphate carboxylase/oxygenase (Rubisco) in efficient CO$_2$ capture as discussed in the earlier sections. An important aspect of all CCMs is the critical roles that the various intracellular and extracellular CAs play in the CCM. In the following section emphasis will be placed on the functional roles of CAs in different photosynthetic CCMs along with an overview of photosynthesis and different CCMs that exist in plants, cyanobacteria and algae.

1. **Effect of CO$_2$ and Presence of a Carbonic Anhydrase on Photosynthesis**

Ambient air contains about 0.036% CO$_2$ and 21% O$_2$. In terrestrial plants, CO$_2$ from the atmosphere enters the leaves through the stomata and diffuses through the substomatal chamber, the intercellular spaces and enters into the mesophyll cells to reach the carboxylation site of Rubisco, which is located in the stroma of the chloroplast. Since CO$_2$ has to diffuse from the
external environment to reach the carboxylation site, the efficiency of carboxylation depends on
the diffusion rate of CO$_2$. The rate of CO$_2$ diffusion is $10^4$ times slower in water than in air. As
stated earlier, the chloroplast stroma of mesophyll cells of higher plants contain about 9 µM CO$_2$
and about 250 µM O$_2$ (Keys, 1986) at 25°C. In aquatic environments, the concentrations of CO$_2$
and O$_2$ can vary from 0 to 250 µM and 0 to 500 µM, respectively (Bowes and Salvucci, 1989).
These estimates underscore the variation in the concentrations of CO$_2$ faced by aquatic
photoautotrophic organisms.

Studies in different species of higher plants have revealed that the C$_3$ plants had
significant increases in dry mass when the concentration of CO$_2$ was increased compared to the
C$_4$ plants grown under the same conditions (Akita and Tananka, 1973). This indicates that many
photoautotrophic organisms operate under conditions in which their photosynthetic rates are
limited by the rate of carboxylation reaction. This suggests that the efficiency of carbon fixation
during photosynthesis may be improved by a Rubisco that is catalytically more active (Andrews
and Lorimer, 1987), by increasing the substrate specificity of Rubisco for CO$_2$ or by elevating
the concentration of CO$_2$ at the site of Rubisco activity.

Most studies have shown that since the control of photosynthesis is shared by several
components of the system under ambient conditions, simply increasing the catalytic activity of
Rubisco would probably not increase the photosynthetic rate. The specificity for CO$_2$ does vary
among different species. The specificity for CO$_2$ is higher in L$_8$S$_8$ forms than in L$_2$ forms and is
highest in Rubisco from higher plants. Intense selection pressure and extensive mutagenesis
experiments have failed to produce a more discriminant form of the enzyme. This suggests that
modifications to eliminate or reduce oxygenations involve multiple changes in the enzyme which
were not favored by evolutionary changes and would not occur in mutagenesis experiments
(Andrews and Lorimer, 1987). The fact that inorganic carbon concentrating mechanisms have
evolved at different instances, implies that these mechanisms probably provide a most cost-effective means of improving photosynthetic rates.

β-CA is present in the chloroplast of mesophyll cells in C₃ plants. Potential roles of the β-CA present in the chloroplast of C₃ plants range from modulation of the pH of stroma to facilitating diffusion of CO₂ across the chloroplast envelope. It is also proposed that the β-CA replenishes the CO₂ supply in the stroma from HCO₃⁻ which is more abundant in the alkaline stroma.

Transgenic tobacco plants were made containing antisense CA constructs by two different groups, to study the function of the β-CA. One group reported no deleterious effects of the reduction of CA activity in the mutant (Price et al., 1994). The other group reported that the antisense plants compensated for the decrease in CA with an increase in stomatal conductance leading to an increase in water loss (Majeau et al., 1994). The reasons for the drought sensitivity are not clear although it is possible that a reduction in photosynthesis due to a decrease in the delivery rate of CO₂ to Rubisco caused the leaf stomata to remain open. It is also possible that lowering the chloroplast β-CA somehow resulted in a disruption of the signal for the plant to close the stomata under certain conditions.

Badger and Pfanz, in 1995, demonstrated the crucial dependence of C₄ photosynthesis on the β-CA activity in the mesophyll cells by using CA inhibitors. In Arabidopsis there is a report that shows that the β-CA is necessary for photosynthesis. Antisense transgenic Arabidopsis plants showing 90% reduction of the β-CA activity were generated by Hee Jin Kim (1997). These antisense CA plants resembled wild type plants when grown either under high CO₂ (2%) conditions or on Murashige-Skoog (MS) containing 2% sucrose (Hee Jin Kim, 1997). When grown in ambient CO₂ conditions (0.03%) or on sucrose free media, most antisense CA plants died. It is possible that the chloroplast β-CA played a more critical role in photosynthesis
during the early Cretaceous era when CO$_2$ concentrations were lower than they are today. The increase of CO$_2$ in the atmosphere may have rendered the chloroplast β-CA expendable except when CO$_2$ is limiting.

2. CO$_2$ Concentrating Mechanism (CCM)

A number of photosynthetic organisms have developed mechanisms to increase the level of CO$_2$ at the location of Rubisco, minimize the energy consuming deleterious oxygenation reaction and reduce nitrogen allocation cost in the form of Rubisco. These include C$_4$ photosynthesis (Hatch, 1987) and Crassulacean Acid Metabolism (CAM), seen in a number of higher plant families and a CO$_2$ concentrating mechanism seen in some microalgae (Osterlind, 1950; Berry et al., 1976) and cyanobacteria (Turpin et al., 1984; Price et al., 1992).

These photosynthetic organisms package Rubisco in a very specific location, have novel means to concentrate CO$_2$ and/or HCO$_3^-$, have a means of rapidly converting the accumulated HCO$_3^-$ to CO$_2$ if HCO$_3^-$ is concentrated and have mechanisms to deliver the CO$_2$ to the location of Rubisco. Environments habituated by many photosynthetic bacteria as well as micro and macro algae, show great fluctuations in concentrations of gases. Many of these organisms employ a mechanism of concentrating CO$_2$ that is induced only under low CO$_2$ conditions (0.036% CO$_2$ in air) and does not operate when high CO$_2$ conditions are prevalent (Osterlind, 1950; Berry et al., 1976). A C$_4$ type mechanism has not been discovered in these organisms and the Rubisco from these organisms have a high $K_m$ for CO$_2$ (Jordan and Ogren, 1981) implying the presence of a novel CO$_2$ concentrating mechanism. The CCM provides these organisms with another additional ecological advantage other than that shared by terrestrial C$_4$ and CAM plants. CCM allows both CO$_2$ and HCO$_3^-$ species to be efficiently exploited. The utilization of HCO$_3^-$ is an advantage as in neutral and alkaline environments HCO$_3^-$ is the most abundant form of inorganic carbon (C$_i$).
All the CO₂ concentrating mechanisms consist of the following components (Badger, 1987):

a) A means to transport inorganic carbon species against a concentration gradient across either the plasma membrane or the chloroplast envelope or both.

b) A source of energy supply to drive the uphill inorganic carbon transport.

c) A compartment where Rubisco is localized separately from the reactions that accumulate the intermediate Ci pool. For C₄ plants this is the bundle sheath, for cyanobacteria it is the carboxysome and for algae it appears to be the pyrenoid.

d) A means for releasing CO₂ from the captured pool, co-localized with Rubisco in the special compartment. This might be decarboxylating enzymes like NAD⁺/NADP⁺ malic enzyme as in C₄ plants and CAs in cyanobacteria and algae.

e) A mechanism to prevent CO₂ efflux from the site of Rubisco to ensure efficient CO₂ fixation.

3. Cyanobacterial CO₂ Concentrating Mechanism

Cyanobacteria (blue green algae) have evolved a remarkable environmental adaptation for survival at limiting CO₂ conditions. This adaptation is known as a CO₂ concentrating mechanism (CCM). A distinguishing feature of the cyanobacterial CCM relative to CCMs in other aquatic organisms is the existence of a constitutive form of CCM in cells grown even at hyper-normal levels of CO₂ (2-5% of CO₂). These high Cᵢ cells have the ability to use CO₂ and HCO₃⁻ as substrates and to accumulate significant levels of Cᵢ with a relatively high photosynthetic affinity for Cᵢ transport.

Cyanobacterial cells exposed to Cᵢ limitation (typically 20-50 ppm CO₂) and air (350 ppm CO₂) have the ability to express an enhanced level of CCM activity. This change is accompanied by an increase in Rubisco activity (Price et al., 1992), about a two fold increase in carboxysome content (McKay et al., 1993; Turpin et al., 1984), transcriptional upregulation of
transport activities and increases in the affinities for CO$_2$ and HCO$_3^-$ uptake activities (Badger and Price, 1992; Kaplan and Reinhold 1999; Kaplan et al., 1994; Price et al., 1998). Typically a 20 fold decrease in the $K_m$ ($C_i$) is seen when cells are grown at 20-30 ppm CO$_2$ levels and more than a half of this rise in affinity is due to an increase in the affinity for HCO$_3^-$ uptake (Sültemeyer et al., 1995; Yu et al., 1994).

Most of the studies of physiological and molecular aspects of the cyanobacterial CCM have been done on the freshwater cyanobacterium *Synechococcus* PCC7942. This is largely because these strains are easily grown in the laboratory under the desirable defined conditions of rapid aeration and high light and their excellent suitability for genetic modifications using recombinant technologies. With the availability of the *Synechosystis* PCC6803 genomic database, considerable attention has been turned to this species as a model for CCM studies. Basic features of the cyanobacterial CCM include the following (Fig. 1.4):

a) Existence of at least four distinct modes of active uptake for CO$_2$ and HCO$_3^-$, with two transport activities being constitutive and another two being inducible by growth under $C_i$ limitation.

b) Irrespective of the type of uptake, there is a resultant accumulation of HCO$_3^-$ within the cells that can be as high as thousand fold with respect to total exogenous levels.

c) Accumulated HCO$_3^-$ is used to increase the CO$_2$ concentration at the site of Rubisco, which is encapsulated in a unique micro-compartment called the carboxysome.

d) The carboxysome contains a specific carbonic anhydrase for conversion of HCO$_3^-$ to CO$_2$ at a rate high enough to match the maximal rate of CO$_2$ fixation.

e) Existence of an effective mechanism for minimizing the leakage of CO$_2$ from the site of elevation in the carboxysome. This mechanism is supplemented by an efficient CO$_2$ uptake mechanism that can recycle leaked CO$_2$ back into the cell as HCO$_3^-$. 

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a. Cyanobacterial Transport Systems for $\text{C}_i$

The model cyanobacterium *Synechococcus* PCC7942 possesses at least four distinct modes of $\text{C}_i$ uptake when grown under $\text{C}_i$ limitation, each possessing a high degree of functional redundancy. Two of these modes of $\text{Ci}$ uptake are induced under low CO$_2$ conditions and the remaining two are constitutively expressed. These four $\text{C}_i$ uptake modes are:

a) The inducible high affinity, Na$^+$ independent HCO$_3^-$ transporter, BCT1 was the first cyanobacterial $\text{C}_i$ uniporter to be identified and characterized. In *Synechococcus* sp., BCT1 is encoded by the $\text{cmpABCD}$ operon and is expressed under $\text{C}_i$ limitation (Omata *et al*., 1999). The deletion mutant, $\text{cmpAB}^{-}$ (M42) has a low affinity for HCO$_3^-$ (Okamura *et al*., 1997). BCT1 is a member of the diverse subfamily of bacterial ABC (ATP binding cassette) (Higgins, 1992) transporters. The $\text{cmpA}$ codes for the precursor of the 42 kDa, HCO$_3^-$ binding protein (cmpA) which is closely related to NrtA (Omata, 1991), a nitrate/nitrite binding lipoprotein for the nitrate/nitrite transporter ($\text{nrtABCD}$) in *Synechococcus* PCC7942.

b) Cells of *Synechococcus* PCC7942 also possess a Na$^+$-dependent HCO$_3^-$ uptake activity. It has been suggested that *Synechococcus* PCC7942 cells may possess a Na$^+/$/HCO$_3^-$ symporter that is energized by inwardly directed Na$^+$ gradient (Espie and Kandasamy, 1994). An indirect role of Na$^+$ through a need for pH regulation via Na$^+$/H$^+$ antiport mechanism is also possible. A gene ($\text{ictB}$) has been suggested as a candidate for constitutive Na$^+$-dependent HCO$_3^-$ transporter as a result of analysis of a HCR mutant in *Synechococcus* that harbors a complex tag mutation. The gene codes for a hypothetical membrane protein with nine to ten membrane spanning domains. Another HCR mutant in *Synechococcus* has a lesion in the gene $\text{dc14}$ (Ronen-Tarazi *et al*., 1998), which encodes a putative Na$^+$-dependent HCO$_3^-$ transporter. This transporter may be involved in a fast induction response to low CO$_2$ in cyanobacteria (Sülttemeyer *et al*., 1997). Recently a gene from *Synechocystis* PCC6803 has been isolated that appears to code for a Na$^+$-
dependent bicarbonate transporter (Shibata et al., 2002). The gene is known as *sbtA* (SLR 1512) and a mutant with lesions in this gene is unable to grow under low HCO$_3^-$ at pH 7 or 9 (Shibata et al., 2002). The *sbtA* protein has 8 to 10 membrane spanning domains and a hydrophobic domain near the center of the protein that may represent a membrane extrinsic region.

c) and d) There are two CO$_2$ uptake systems based on specialized forms of thylakoid-based Type 1 NADPH dehydrogenase complexes (NDH-1). One of the CO$_2$ uptake systems is constitutive and uses a specialized form of the thylakoid based, Type 1 NADPH complex referred to as NDH-1$_4$, with specific subunits coded by *NDHF4*, *ChpX* and *NDHD4*. The other CO$_2$ uptake system referred to as NDH-1$_3$, is induced under low CO$_2$ conditions and has subunits coded by *NDHF3*- *NDHD3*-*ChpY* operon. The two unique proteins ChpX and ChpY (also called CupA and CupB) (Maeda et al., 2001; Shibata et al., 2001) have CO$_2$ hydration activity in the light. They have no sequence homology with the known families of CA proteins. However, ChpX has two conserved histidine and one conserved cysteine residues in the highly conserved region in the protein. These conserved residues could be potential coordination sites for a Zn atom in the active site. ChpX is bound to the NDH-1 complex and is in close contact with NDHF4 and NDHD4 so as to create a “proton wire” for abstraction of protons to the thylakoid lumen via the photosynthetic electron transport chain. ChpY can function in a similar way (Price et al., 2003). Thus C$_i$ uptake is tightly light dependent.

b. Rubisco Localization and the Role of the Carboxysome in CCM in Cyanobacteria

In cyanobacteria (blue green algae), a number of alterations in the structure, localization and conformation of Rubisco have been shown to cause HCR mutants. Immunogold labeling shows Rubisco is localized to the micro compartment called the carboxysome (McKay et al., 1993). Carboxysomes are proteinaceous, semicrystalline bodies surrounded by a proteinaceous shell. C$_i$ is delivered into the cell in the form of HCO$_3^-$ and remains largely as HCO$_3^-$ except in
Figure 1.4 A proposed model for CCM in cyanobacteria. The font sizes of CO$_2$ and HCO$_3^-$ indicate the relative concentration of these Ci species.
the carboxysome where a β-CA converts the HCO$_3^-$ to CO$_2$ (Badger and Price, 1992). This increases the concentration of CO$_2$ at the site of Rubisco, ensuring efficient CO$_2$ fixation.

The carboxysomal CA has a 60-70 amino acid extension at the carboxyl end of the protein (Badger and Price, 1994) when compared with other prokaryotic β-CAs giving a monomer size of 31 kDa compared to 24 kDa for the chloroplast enzyme of higher plants. The proximal part of the carboxyl extension may be required for the oligomerisation of the CA and this oligomerization is essential for catalysis (So et al., 2002).

Recent genome analysis of seven cyanobacterial genomes has lead to the identification of two types of carboxysomes in cyanobacteria (Badger, 2003). One type has the typical cyanobacterial carboxysomes and the other type has carboxysomes similar to autotrophic β-proteobacteria like *Thiobacillus* species. In one marine cyanobacterium *Synechococcus* WH8102, the CA does not have the carboxyl terminal extension found in other carboxysomal CAs (Badger et al., 2002). Moreover, *Prochlorococcus marinus* MED4 and MIT9313 have no identifiable CAs in their genome (Badger et al., 2002). This clearly raises questions about the presence of carboxysomal CA in this type of carboxysome and also about the roles of CAs in general in these species. It also asks if yet to be identified CAs, are present in these organisms.

To prevent the dissipation of the HCO$_3^-$ pool, CA activity should be absent in the cytosol of cyanobacteria. The expression of human CA in the cytoplasm of *Synechococcus* PCC7942 cells results in a massive leakage of CO$_2$ from the cells producing a HCR phenotype and implicating the carboxysomes as the site of CO$_2$ elevation (Price and Badger, 1989a). Also the cyanobacterial genome sequences do not appear to code for a CA that is expressed in the cytosol (Badger et al., 2002). A cluster of five genes, *ccmKLMNO*, have been shown to be involved in structural assembly or functioning of carboxysome. Mutations in some of these genes (*ccmA* and *ccmJ*) produce mutant cells that have HCR phenotypes and are unable to utilize the intracellular
Ci pool although they are capable of C\textsubscript{i} transport (Badger and Price, 1994; Ronen-Tarazi, \textit{et al.}, 1995).

The \textit{CcmM} gene codes for the \(\gamma\)-CA analog, CcmM. Cells deleted in \textit{CcmM} show HCR phenotype and have empty carboxysomes. From these results it is clear that CcmM is required for correct carboxysome assembly and for optimal growth on low levels of CO\textsubscript{2}. It is not clear, if CcmM has CA activity or its enzymatic activity is needed for correct assembly of carboxysomes. CO\textsubscript{2} leakage out of the carboxysome has been hypothesized to be prevented by the spatial arrangement of CA and Rubisco in the carboxysomes (Reinhold \textit{et al.}, 1991), which ensures that the CO\textsubscript{2} produced is fixed before it can leak to the cytoplasm. CO\textsubscript{2} leakage is further minimized by the carboxysomal proteinaceous shell which is selectively permeable to HCO\textsubscript{3}\textsuperscript{-} and not to CO\textsubscript{2}.

Characterization of a \textit{Synechococcus} mutant containing a disruption in an ORF (ORF 272) led to the isolation of the carboxysomal \(\beta\)-CA gene \textit{icfA} and its product (Fukuzawa \textit{et al.}, 1992; Yu \textit{et al.}, 1992). The loss of this CA leads to a cell that cannot grow very well on limiting levels of CO\textsubscript{2}. This mutant accumulates HCO\textsubscript{3}\textsuperscript{-} to higher levels than wild type cells, presumably because these cells can no longer convert HCO\textsubscript{3}\textsuperscript{-} to CO\textsubscript{2}. This CA is co-localized in the carboxysome with Rubisco (Price \textit{et al.}, 1992).

Replacement of Rubisco of \textit{Synechocystis} PCC6803 with that of \textit{Rhodospirillum rubrum}, led to the loss of carboxysomes and the mutant showed a HCR phenotype, although it was capable of accumulating C\textsubscript{i}. Studies indicated that this \textit{Rhodospirillum} Rubisco was not packaged into the carboxysome. \textit{Rhodospirillum} Rubisco lacks small subunits and has poor specificity for CO\textsubscript{2} versus O\textsubscript{2}. In cyanobacteria the small subunits might play a role in the structural organization of the carboxysome.

Characterization of one cyanobacterial HCR mutant, EK6, showed that it contains a thirty amino acid extension in the Rubisco small subunit (Orús \textit{et al.}, 1995). This resulted in a Rubisco
that was incapable of packing into the carboxysome. *In vitro* analysis showed that this Rubisco has the same $K_m$ (CO$_2$) as the wild type enzyme (Schwarz et al., 1995). Another HCR mutant, Mu28 (Friedberg et al., 1993), does not have any apparent carboxysomes and immunolabelling studies showed that the Rubisco is distributed throughout the mutant cell while in wild type cell it was localized in the carboxysome.

**c. Other Genes and Proteins Involved in the Cyanobacterial CCM**

A number of other genes affect the $C_i$ accumulation mechanism in cyanobacteria, apart from genes contributing to Rubisco, carboxysome and NADH dehydrogenase complex (Price et al., 1998). These genes include:

1) **$RbcR$** There are two copies of the putative transcription regulator gene $RbcR$ ($RbcR1$ and $RbcR2$) in *Synechocystis* PCC6803 genome. Loss of induction of the *cmp* operon occurs at low CO$_2$ conditions when $RbcR1$ is inactivated. This indicates that the $RbcR1$ regulates transcription under $C_i$ limitation.

2) **icfG** In *Synechocystis*, the gene *icfG* has been implicated in the down regulation of $C_i$ uptake ability. *icfG* is expressed in the presence of glucose and is required for the switch from photoautotrophic to photoheterotrophic growth (Beuf et al., 1994).

3) **SLR0143** This gene codes for a large protein with similarity to a beta transducin like protein. Mutation in this gene results in HCR phenotype with reduced affinity for $C_i$ uptake (Bédu et al., 1995).

4) **cotA** It codes for a membrane protein that has a haem binding site similar to chloroplast envelope protein $CemA$ (Katoh et al. 1996). *CotA* mutants have reduced $C_i$ uptake and reduced H$^+$ extrusion during initial uptake phase. This mutant does not grow at low Na$^+$ conditions at neutral pH but can grow at high pH even at low Na$^+$. It is speculated that this protein helps in proton extrusion from the cell during HCO$_3^-$ uptake.
5) *ecaA* This gene codes for a periplasmic α-CA in *Synechococcus* sp. and *Anabaena* PCC7120 (Soltes-Rak *et al*., 1997). This gene is upregulated under high CO₂ conditions and downregulated under low CO₂ conditions. Insertional inactivation of this gene did not produce a clear phenotype so whether or not the activity of this α-CA facilitates the supply of CO₂ to the plasma membrane remains unclear. It is likely that it facilitates the diffusion of CO₂ across the plasma membrane. *Synechocystis* PCC6803 lacks this α-CA.

4. **CO₂ Concentrating Mechanism in Chlamydomonas**

The ability of unicellular green algae to grow well both under low and high CO₂ conditions was first discovered by Osterlind (1950). It has been proposed that unicellular green algae grown under limiting levels of CO₂ acquire the ability to utilize CO₂ more efficiently (Berry *et al*., 1976). A specific inducible CCM has been suggested to explain this phenomenon (Badger *et al*., 1980; Aizawa and Miyachi, 1986; Badger and Price, 1992). This low CO₂ inducible CCM does not operate in high CO₂ grown cells. The main function of this CCM is to elevate the CO₂ concentration at the site of Rubisco. Ci accumulation has been observed in many algal species including red algae (Burns and Beardall, 1987), diatoms (Coleman and Rotatore, 1995), marine green algae (Burns and Beardall, 1987; Beer *et al*., 1990) fresh water algae symbiotic algae (zooxanthellae) and dinoflagellates (Berman-Frank *et al*., 1998; Leggat *et al*., 1999). When grown under high CO₂ conditions (3%-5% CO₂ in air) these organisms show low affinity for CO₂ and high rates of photorespiration. Under low CO₂ conditions (0.035%), these organisms show high affinity for CO₂, low rates of photorespiration and an ability to concentrate Cᵢ at a level higher than that in the surrounding environment. This CCM mechanism is different from the C₄ mechanism of CO₂ concentration. The algal CCM is different from the cyanobacterial CCM, probably due to the reason that the prokaryotic cells have one set of limiting membranes while the eukaryotic green algae have two sets of membranes for Cᵢ to cross.
a. The Proposed Model

Several CCM models have been proposed (Moroney et al., 1986; Moroney and Mason, 1991; Badger and Price 1994). Moroney and Mason (1991) proposed the model shown in Fig. 1.5. According to this model, a CA-promoted diffusion of CO₂ occurs across the plasma membrane as seen in *C. reinhardtii* and *Chlorella saccharophila* strains. Under low CO₂ conditions, these algae accumulate high concentrations of periplasmic CA (Kimpel et al., 1983). CO₂ diffuses across the plasma membrane into the cytoplasm. In the cytoplasm, this CO₂ is converted into HCO₃⁻ by either an uncatalyzed reaction or in the presence of a cytoplasmic CA. This HCO₃⁻ in the cytoplasm is then actively transported into the chloroplast. In the chloroplast, HCO₃⁻ diffuses to the pyrenoid. The pyrenoid is a large protein complex surrounded by a sheath of carbohydrates like starch, amylose or paramylon (Gibbs, 1962; Okada, 1992). The majority of the Rubisco is localized in the pyrenoid in algae. Another CA, which may be located in the chloroplast envelope or thylakoid or in the pyrenoid, catalyzes the conversion of HCO₃⁻ to CO₂. This CO₂ can diffuse to the site of Rubisco. This elevation of CO₂ at the site of Rubisco reduces the oxygenation reaction of Rubisco.

b. Physiological Studies

Evidence for CO₂ as the inorganic carbon species crossing the plasma membrane in *C. reinhardtii* comes from experiments using C₅ as a substrate for fixation studies (Tsuzuki, 1983), the effect of external pH on CO₂ fixation (Moroney and Tolbert, 1985) and from the use of impermeant inhibitors of CAs like acetazolamide (AZ) (Moroney et al., 1985). CO₂ fixation and C₅ accumulation were inhibited by AZ (Moroney et al., 1985). This shows HCO₃⁻ is not the Ci species transported across the plasma membrane. Organisms growing in acidic conditions like *C. saccharophila*, can take up only CO₂ while those growing in alkaline conditions can take up both CO₂ and HCO₃⁻ (Beardall, 1981). Active uptake of HCO₃⁻ has been suggested in the green alga
Figure 1.5 A model showing the potential roles of carbonic anhydrases in the operation of CCM in *C. reinhardtii*. The font sizes of CO₂ and HCO₃⁻ indicate the relative concentration of these Cᵢ species. cCA and cy CA represent chloroplastic and cytoplasmic carbonic anhydrases. Cah1 and Cah3 represent the periplasmic and thylakoid CA, respectively. Putative HCO₃⁻ transporters are denoted by brown small circles.
Scenedesmus (Thielmann et al., 1990) because C_i transport in this alga is not affected by AZ. This indicates that the organism does not need a CA in the periplasm.

An active transport has also been proposed in C. reinhardtii (Sütemeyer et al., 1989). Transport activities for CO_2 have been found on the chloroplast envelope (Amoroso et al., 1998) and there is also evidence for HCO_3^- uptake across the cell membrane (Palmqvist et al., 1994; Amoroso et al., 1998). However, to date, CO_2 and/or HCO_3^- transporters at the plasma membrane or chloroplast envelope have not been identified. The NdhF, NdhD and Chp subunits associated with the NDH-1 complex in blue green algae (cyanobacteria) have no homology in the EST database of C. reinhardtii. The argument that active transport at the plasma membrane is not the main transport step for C_i is strengthened by the evidence that isolated chloroplasts can concentrate CO_2 at low CO_2 conditions (Moroney et al., 1987; Goyal and Tolbert, 1988). When grown under high CO_2 conditions, green algal cells behave like C_3 plants, producing large amounts of photorespiratory products and showing a K_m (CO_2) of 20 µM (Moroney and Tolbert, 1985). On acclimation to low CO_2 conditions, the K_m (CO_2) decreases to 2 µM along with a significant reduction of photorespiratory byproducts. Low CO_2 grown cells accumulate acid-labile ^14C, in the presence of light and NaH^14CO_3. This implies that there is an increase in the concentration of intracellular C_i. C_i accumulation can be blocked by inhibitors of electron transport or photophosphorylation (Badger and Price 1994; Berry et al., 1976). This indicates that light energy establishes and maintains this inorganic carbon concentrating mechanism.

c. Low CO_2 Inducible Proteins and Genes in Chlamydomonas reinhardtii

When CCM is induced under low CO_2 conditions, the algal cells preferentially synthesize some new proteins from newly transcribed mRNAs. Based on the inducibility of CCM, two approaches have been employed to identify the components involved in this mechanism. One method involves the identification, purification, sequencing and characterization of the
newly synthesized polypeptides. The second involves the construction of a cDNA library using mRNA from low and high CO\textsubscript{2} adapted cells. These cDNA libraries could then be differentially screened for cDNAs induced or unregulated under low CO\textsubscript{2} conditions. A description of some of these proteins and genes are given below.

Based on physiological studies, it can be assumed that the proteins that are involved in CCM are induced or derepressed only on adaptation to low CO\textsubscript{2} conditions (Badger et al., 1980). \textit{In vivo} labeling studies using \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2-} demonstrated that five polypeptides of molecular weights 46, 44, 37, 36 and 20 kDa are preferentially synthesized in \textit{C. reinhardtii} under low CO\textsubscript{2} conditions (Manuel and Moroney, 1988). The 37 kDa protein has been identified as a periplasmic CA (Cah1) (Coleman and Grossman, 1984; Fukuzawa et al., 1990). There are two periplasmic \(\alpha\)-CA genes, namely \textit{Cah1} and \textit{Cah2}. \textit{Cah1} is required for the functional operation of CCM in \textit{C. reinhardtii} (Moroney et al., 1985). \textit{Cah1} facilitates the diffusion of both CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} to the cell surface by promoting rapid equilibrium. CO\textsubscript{2} is then available for both passive diffusion and active uptake across the cell membrane and HCO\textsubscript{3}\textsuperscript{-} is available for HCO\textsubscript{3}\textsuperscript{-} uptake systems. The concept that HCO\textsubscript{3}\textsuperscript{-} is the main Ci species that is transported across the plasma membrane is supported by the observation that CA inhibitor AZ inhibits CO\textsubscript{2} fixation at high pHs where HCO\textsubscript{3}\textsuperscript{-} is predominant (Moroney et al., 1985). Recent studies have cast some doubt on these concepts when a \textit{Chlamydomonas} mutant defective in \textit{Cah1} gene appeared to perform as well as wild type cells at limiting C\textsubscript{i} conditions (Van and Spalding, 1999). An explanation for these conflicting results may lie in the fact that the physiology was tested at near pH 7 where HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2} are almost equal in abundance. It has been suggested that the defective phenotype may be only apparent at high pHs or that an increased contribution from the \textit{Cah2} may alleviate potential limitations (Moroney et al., 2001). This high pH condition can be found where unstirred boundary layer conditions are high such as in algal films rather than in dilute
liquid culture. Cah2 is not expressed under low CO₂ conditions and is never expressed at high levels. The function of Cah2 is not clear.

Other proteins upregulated under low CO₂ condition include a 53 kDa alanine aminotransferase (Chen et al., 1996) and two 21 kDa mitochondrial β-CAs (Karlsson et al., 1995). During photorespiration CO₂ is produced. CO₂ inhibits the mitochondrial respiration (González-Meler et al., 1996) and this mitochondrial CA could be involved in the efflux of this CO₂. This CA can stabilize the pH in the mitochondria during photorespiration by increasing the rate at which photorespiratory NH₃ is converted to NH₄⁺ with uptake of one H⁺ from the mitochondrial matrix (Eriksson et al., 1998). It has been suggested that these mitochondrial CAs play a role in recycling both respiratory and photorespiratory CO₂ by converting it to HCO₃⁻ in the mitochondrial matrix (Raven, 2001). The HCO₃⁻ would leak back into the cytosol where it would be available for transport into the chloroplast. This model assumes the absence of CA from the cytosol. Recently, it has been shown that the expressions of these mitochondrial CAs decrease when the external NH₄⁺ concentration decreases, to the point of being undetectable when the NH₄⁺ supply restricts the rate of photoautotrophic growth (Giordano et al., 2003). The expression of these CAs were induced at 0.2% CO₂ condition by increasing the NH₄⁺ condition in the growth medium. The workers have proposed that the mitochondrial CAs are involved in supplying HCO₃⁻ for anaplerotic assimilation catalyzed by phosphoenolpyruvate carboxylase, which provides carbon skeletons for nitrogen assimilation under certain conditions.

Two other polypeptides (45 kDa and 42 kDa) are expressed under low CO₂ conditions and their identities are still not clear. Absence of any of these proteins may be correlated to an inability for growth under low CO₂ conditions and indicate that these proteins play a role in CCM. The 36 kDa protein (LIP-36) identified in the labeling studies and subsequently purified (Spalding and Jeffrey, 1989; Geraghty et al., 1990) has been localized specifically to chloroplast
envelope membranes (Mason et al., 1990; Ramazanov et al., 1993). It has been shown to be encoded by two genes and the protein sequence shows homology to a mitochondrial carrier protein superfamily (Chen et al., 1996).

Analysis of the CCM mutant \textit{pmp1}, shows that it synthesizes all the known low CO$_2$ inducible polypeptides under low CO$_2$ conditions except the 42 and 45 kDa proteins. This mutant accumulates $C_i$ but to levels lower than the wild type. Another mutant, \textit{cia5}, has a HCR phenotype, fails to induce CCM and does not synthesize any of the low CO$_2$ inducible polypeptides. It is deficient in a putative transcription factor (Fukuzawa et al., 2001; Xiang, \textit{et al}, 2001).

d. Pyrenoid, Rubisco Localization and Its Role in CCM

The pyrenoid, thought to be the structural equivalent of the carboxysome, is found in most eukaryotic green algae. Pyrenoids have been purified from both \textit{Eremosperma} (Okada, 1992) and \textit{C. reinhardtii} (Kuchitsu \textit{et al}, 1991) and in both cases they consisted primarily of Rubisco. In addition, \textit{C. reinhardtii} cells with a mutation in the rbcL gene (Rubisco large subunit) that leads to a truncation of the large subunit of Rubisco have no pyrenoids (Rawat \textit{et al}, 1996). Although it is accepted that Rubisco is the major constituent of the pyrenoid, there are conflicting findings regarding what percentage of the cell’s Rubisco is in the pyrenoid. Cryofixation and immunogold studies have indicated that the pyrenoid in the low CO$_2$ grown cells contain almost 90% of the Rubisco (McKay and Gibbs, 1991; McKay \textit{et al}, 1991; Morita \textit{et al}, 1997; Borkhsenious \textit{et al}, 1998). \textit{In vitro} measurements of Rubisco activity imply that the enzyme in the pyrenoid must be active to account for the CO$_2$ fixation rate observed in \textit{C. reinhardtii}. Under high CO$_2$ conditions only 50% of the Rubisco is found in the pyrenoid (Borkhsenious \textit{et al}, 1998). In lichens and bryophytes there is a good correlation between the operation of a CCM and the presence of a pyrenoid (Smith and Griffiths, 1996). A specific
location of Rubisco is also compatible with the view that organisms that have CCMs specifically package Rubisco. In *C. reinhardtii*, a rapid formation of starch sheath has been observed in response to low CO$_2$. This pyrenoid starch sheath formation is partially inhibited by the presence of acetate in the growth medium under light and low CO$_2$ conditions. When cells are grown in the dark, the CCM is not induced and the starch sheath is not formed even though there is a large amount of starch accumulation in the chloroplast stroma. The membrane permeant CA inhibitor EZ inhibits formation of the starch sheath under low CO$_2$ conditions (Ramazanov et al., 1996). Some of the HCR mutants partially or fully lack the starch sheath. These observations suggest that the ultrastructural reorganization of the pyrenoid starch sheath under low CO$_2$ conditions may play a role similar to the carboxysome in cyanobacteria (Borkhensive et al., 1998).

The thylakoid $\alpha$-CA (Cah3) appears to be targeted to the thylakoid lumen and is associated with PSII (Karlsson et al., 1998; Villarejo et al., 2002; Park et al., 1999). A *C. reinhardtii* mutant *cia3*, that has two point mutations in the region coding for the transit peptide of the Cah3 protein, grows poorly under low CO$_2$ but grows fine under high CO$_2$ (Fig. 1.6). ca-1, another allelic mutant of Cah3 showed the same phenotype under low CO$_2$. The sequence of the *ca-1* gene revealed a point mutation in the 5´ end of the gene that created a stop codon. *cia3* and *ca-1 allelic* mutants were complemented by the wild type *Cah3* gene (Karlsson et al., 1998; Funke et al., 1997) and the transformants resembled wild type cells and grew fine under low CO$_2$. This shows that Cah3 is required for the growth of *Chlamydomonas* in air levels of CO$_2$ (Funke et al., 1997).

It had been initially suggested that this CA speeds up the formation of CO$_2$ from HCO$_3^-$ in the acidic lumen of thylakoids and that this CO$_2$ diffuses through the thylakoid membrane to the pyrenoid (Moroney and Mason, 1991; Badger and Price, 1994; Raven, 1997; Moroney and Somanchi, 1999). This model is based on the assumption that HCO$_3^-$ is actively pumped into the
Figure 1.6  Growth of the wild type and the cah3 mutant cells of C. reinhardtii on the minimal media under low and high CO₂ conditions. ca-1 and cia3 are allelic cah3 mutants. WT2137 is the parent of ca-1 mutant strain and WT137 is the parent of cia3 mutant strain.
lumen from the stroma and also on the fact that in many algae, including *Chlamydomonas*, thylakoids penetrate the pyrenoid matrix in various patterns (Raven, 1997).

It has also been proposed that the activity of this CA stabilizes the manganese cluster of PSII and maintains the oxygen evolution complex (OEC) in a functionally active state (Villarejo *et al.*, 2002). In the mutant *cia3*, the OEC contains two manganese atoms per PSII reaction center and shows very low OEC activity. Addition of HCO$_3^-$ or manganese to the thylakoids and BBY (Berthold-Babcock-Yocum PSII particles) preparations from the mutant can restore the wild type OEC activity (Villarejo *et al.*, 2002). Recently, it has been proposed that the thylakoid CA is mainly required for the proper functioning of CCM under low CO$_2$ conditions by providing elevated levels of CO$_2$ to Rubisco. It might have the secondary role of stabilizing PSII at high light, possibly by preventing an excessively low luminal pH through rapid dehydration of HCO$_3^-$ to CO$_2$ and H$_2$O (Hanson *et al.*, 2003). To date, there is no report of a thylakoid localized CA in higher plants. This also seems to implicate that probably Cah3 is not essential for the proper functioning of PSII.

There may be diversity in the ways in which CO$_2$ is generated in the chloroplast and where it is elevated (Raven, 1997; Badger *et al.*, 1998). For example both the pyrenoid and thylakoid lumen based CAs might release CO$_2$ from HCO$_3^-$ pool in the stroma. There may also be a chloroplast envelope CA. The possibility that CO$_2$ may be elevated within the entire chloroplast rather than just the pyrenoid has also been suggested (Badger, 2003).

Thylakoid associated α-CA has been found also in two other green microalgae, *Tetraedron minimum* and *Chlamydomonas noctigama* (van Hunnik *et al.*, 2001). Some arctic species of unicellular green algae such as *Chloromonas* and the lichen photobionts like *Coccomyxa* lack both pyrenoids and the CCM (Palmqvist *et al.*, 1994; Morita *et al.*, 1997). Therefore it is speculated these algae lacking both pyrenoids and CCM may be slow growing.
organisms adapted to environments in which CCM might not be necessary for survival. (Honegger, 1991).

Recent data on the physiology of carbon fixation in the marine diatom *Thalassiosira weissflogii* suggest that a form of C\(_4\) photosynthesis may operate together with active HCO\(_3^-\) uptake (Reinfelder *et al*., 2000). In this respect a novel δ-CA class of enzyme has been identified which is unrelated to other three classes of CAs (Cox *et al*., 2000). This enzyme appears to be localized in the cytosol and presumably could participate in providing HCO\(_3^-\) to PEP carboxylase (Morel *et al*., 2002). However, there is some controversy regarding the data and further investigation will be needed to resolve the picture. Recently a β-CA has been identified in another marine diatom *Phaeodactylum tricornutum* (Satoh *et al*., 2001) although its localization is uncertain.

5. **CCM in Bryophytes**

Bryophytes are among the first green plants to colonize the terrestrial environment. They are closely related to the higher plants that dominate the land today (Nickrent *et al*., 2000). There is less discrimination against \(^{13}\)CO\(_2\) during photosynthetic carbon fixation in bryophytes. This indicates that a carbon concentrating mechanism is present in these plants. Anthocerotophyta (hornworts), a group of Bryophytes, have unique carbon concentrating properties. Many members of this group have one chloroplast per cell and a pyrenoid body is present in the chloroplast. These organisms have increased affinities for CO\(_2\), reduced CO\(_2\) compensation points, accumulate C\(_i\) and to some extent show reduced O\(_2\) sensitivity of photosynthesis (Smith and Griffiths, 1996; Hanson *et al*., 2002). The CCM in these plants is hampered when they are treated by the CA inhibitor EZ (Smith and Griffiths, 2000). This indicates a role for CA similar to other algal chloroplast based CCMs. No information about CAs in these organisms is known.
OTHER FUNCTIONAL ROLES PLAYED BY CAs IN DIFFERENT GROUPS OF ORGANISMS

Carbonic anhydrase has been implicated in many physiological processes such as carboxylation/decarboxylation reactions, transport of CO$_2$ and/or HCO$_3^-$ across membranes either by passive or active mechanisms, pH regulation, ion exchange, calcification, metabolism of urea, glucose and lipids and efficient viral replication. Membrane associated human CAXVI appears to be associated with plasma membrane of cells of the proximal convoluted tubule of the kidney suggesting a role in the reabsorption of HCO$_3^-$ by that organ. Two recently characterized transmembrane proteins, CAIX and CAXII, have been linked to oncogenesis and their overexpression has been observed in malignant tumors (Türeci et al., 1998; Ivanov et al., 1998). Recently a RNA and DNA binding nuclear factor, nonO/p54$^{nh}$ has been shown to possess CA activity (Karhumaa et al., 2000). The activity of nonclassical CA nonO, is higher than CAIII and CAV and may function in the maintenance of pH homeostasis in the nucleus.

Deletion of the β-CA-like gene NCE103 of the yeast Saccharomyces cerevisiae causes an oxygen-sensitive growth defect (Götz et al., 1999). The protein has no detectable CA activity. Expression of the Medicago sativa CA gene in a yeast expression cassette on a multicopy plasmid complemented the growth defects caused by the deletion of the NCE103 gene. This shows that the Nce103 protein is required for protection against oxidative metabolism. Recently it has been found that the tobacco salicylic acid-binding protein 3 (SABP3) is a chloroplast β-CA, which exhibits antioxidant activity and plays a role in the hypersensitive defense response (Slaymaker et al., 2001).

In Escherichia coli, a eubacterium, cyanate induces the expression of cyn operon. The cyn operon includes the gene cynS which encodes a cyanase that catalyzes the reaction of cyanate with HCO$_3^-$ to give ammonia and CO$_2$. The cynT gene which codes for a β-CA, is a part of the
cyn operon. This β-CA recycles the CO₂ produced in the cynase reaction back to HCO₃⁻ which would have diffused out of the cell (Guilloton et al., 1993).

*Methanosarcina thermophila* is a methanogen in that it obtains energy for growth by converting the methyl groups of acetate, methanol or methanolamines to methane. During metabolism of acetate, oxidation of the carbonyl group provides electrons for reduction of the methyl group leaving CO₂ as the second product. A metabolic switch from methanol to acetate elevates γ-CA activity suggesting this enzyme is important for growth on acetate. It has been proposed that CA may be required for a CH₃CO₂⁻/H⁺ symport system or for efficient removal of cytoplasmically produced CO₂ (Alber and Ferry, 1994).

An α-CA has been recently identified throughout young nodules in soybean and mainly in the cortical regions of old nodules (Kavroulakis et al., 2000). This suggests that this CA recycles CO₂ early in the nodule development and facilitates diffusion of CO₂ from the nodule later in development.

There has been an increasing interest in CAs from plants and algae over the past decade. This interest began with the discovery of the β-CA in plants in 1990 (Fawcett et al., 1990) and has continued with the finding of multiple α- and β-CAs in *C. reinhardtii* and *A. thaliana* and the determination of the critical physiological roles CAs have in cyanobacteria and macro-algae. There are still numerous unresolved questions regarding the number and distribution of CA genes and gene families, structure, localization and function. The availability of *Arabidopsis* and *Chlamydomonas* genome sequences can be used to find out the exact number of CA isoforms in these organisms. The challenge for future researchers will be to determine the expression patterns, localization and physiological roles for each of these isoforms. As there appears to be a large number of isoforms in plants and algae, CA researchers have a lot more to work on in the near future.
This dissertation is the report on my efforts to a) identify, clone, and overexpress novel CA and CA-like genes from the green unicellular algae *Chlamydomonas reinhardtii* in *Escherichia coli*, b) partially biochemically characterize the recombinant CA protein if it is active, and c) determine if the new active CA plays any role in photosynthesis and CCM in this green alga.
CHAPTER 2
MATERIALS AND METHODS

CELL CULTURE

Wild type *C. reinhardtii* 137+ and 2137+ were obtained from Dr. R. K. Togasaki, of Indiana University, Bloomington, and from *Chlamydomonas* Genetic Center at Duke University, Durham, respectively. The strain D66 (nit2’, cw15, mt+) obtained from Rogene Schnell, University of Arkansas-Little Rock (Schnell and LeFebvre, 1993) and ca-1 was obtained from Dr. M. Spalding, of Iowa State University, Ames, Iowa. Strains cia3 and the cia3 transformant 9A were generated by Dr. J. V. Moroney’s laboratory group (Moroney *et al*., 1986; Karlsson *et al*., 1998). To start cultures, cells from yeast acetate medium plates were inoculated into 100 mL of TAP (Tris-Acetate-Phosphate) medium (Sueoka, 1960) and grown with continuous shaking and light (300 μmol photons • m⁻² • s⁻¹) for two days. An aliquot of the culture was then transferred to 1.5 L of minimal medium (Sueoka, 1960) and bubbled with high CO₂ (5% CO₂ in air) until it reached a cell density of about 2 x 10⁶ cells mL⁻¹. The culture was diluted with an equal volume of fresh medium and split into two flasks. One was bubbled with high CO₂ and the other with low CO₂ (0.035% CO₂ in air). The time of low CO₂ acclimation varied from 2 to 12 hours. The high and low CO₂ acclimated D66 cells were used for RNA isolation, measurement of chlorophyll content and Western blots. Only low CO₂ acclimated cells of strains D66, 137+, ca-1, cia3 and 9A were used for chlorophyll assays and Western blots.

TOTAL RNA ISOLATION AND NORTHERN BLOT ANALYSES

All glassware used for RNA isolation was either heated to 180°C overnight or soaked with DEPC (diethyl pyrocarbonate)-treated water and autoclaved for two hours. Total RNA was
extracted from C. reinhardtii using standard procedures (Sambrook et al., 1989). Two liters of cultures of either high or low CO2 grown cells were harvested and resuspended in 10 mL of DEPC-treated water in a 150 mL Corex tube. To this, 20 mL of 2X lysis buffer [100 mM Tris-HCl, 4% SDS, 3 M NaCl and 30 mM EDTA (ethylenediaminetetraacetic acid; pH 8); made with DEPC-treated water and autoclaved for one hour] and 3 mg of proteinase K (10 mg mL⁻¹) were added. The final concentration of proteinase K was 100 µg mL⁻¹. The mixture was gently stirred at room temperature for 20 minutes. The cell lysate was extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1,v/v) and once with chloroform. After centrifugation at 2500 rpm in a swinging bucket rotor for 15 minutes, the supernatant was transferred to a clean tube to which an equal volume of isopropanol was added. The tube was kept at -20°C overnight and the RNA was collected by centrifugation at 15000 g for 20 minutes at 4°C.

The RNA pellet was washed once with 80% ethanol and air dried for several hours. The RNA was dissolved in 4 mL of DEPC-treated water. LiCl (12 M) was added to the solution to achieve a final concentration of 2.5 M and the RNA was precipitated overnight at 4°C. RNA was collected by centrifuging at 15000 g for 20 minutes at 4°C. After being washed, the RNA pellet was dried and resuspended in 6 mL of DEPC-treated water. RNA was reprecipitated by adding 2.5 volumes of ethanol at -20°C for 4 hours. The recovered RNA was washed, air dried and resuspended in 1 mL of DEPC-treated water.

All RNA samples were aliquoted (10 µg in each tube) and stored at -70°C. Total RNA concentration was determined by absorbance at 260 nm, using the conversion 1 OD₂₆₀ = 40 µg of RNA mL⁻¹. For Northern blot analyses, 5.5 µL of RNA (20 µg) was added to 1 µL of 10X MOPS (morpholinepropanesulfonic acid) buffer, 3.5 µL of formaldehyde, 10 µL of formamide and incubated at 65°C for 15 minutes. Four µL of loading buffer [50% glycerol, 1 mM EDTA (pH 8), 0.25% bromophenol blue, 0.25% xylene cyanol FF] was added after the samples were
chilled on ice for 3 minutes. The denatured RNA extracted from high or low CO$_2$ adapted cells was resolved on a 1% agarose gel containing 6% formaldehyde [gel running buffer: 1 X MOPS, 6% formaldehyde; 10 X MOPS gel running buffer: 0.2 M MOPS (pH 7.0), 80 mM sodium acetate, 10 mM EDTA; the solution was filter sterilized or autoclaved for 15 minutes]. The gel was soaked overnight in DEPC-treated water and transferred to a BA-S 85 nitrocellulose membrane (Schleicher and Schuell Bioscience Inc, Keene, New Hampshire). The Northern blot was probed with the radiolabeled $Cah6$ PCR product described in the next section. The final washing conditions were 0.5 X SSC + 0.1% SDS at 55°C for 2 hours or longer.

**RANDOM LABELING OF DNA TO PRODUCE THE RADIOACTIVE PROBE**

When $Cah6$ primers X-9 and R5 were used to perform PCR on the cDNA core library, an 826 bp PCR product is generated. This product was used to make a radioactive probe to study the expression pattern of $Cah6$ under low and high CO$_2$ conditions. The PCR product corresponded to an 826 bp region in the 3´ UTR of $Cah6$. This region was selected as it was unique to $Cah6$ and not similar to any sequence in the two mitochondrial β-CAs, present in *C. reinhardtii*. Sequences of the X-9 and R5 primers are given in Appendix 1. One µL of 50 ng µL$^{-1}$ random hexanucleotide primers was mixed with the 50 ng of purified $Cah6$ PCR product in a 0.5 mL centrifuge tube and the final volume was adjusted with water to 10 µL. After the DNA and the primer mixture were boiled for 5 minutes and instantly chilled on ice for 10 minutes, the following components were added to the tube: 2 µL of 10 X DNA I buffer (NEB, Beverly, Massachussets), 2 µL of dNTP (2.5 mM each; no dCTP), 5 µL of $^{32}$P-dCTP (10 µCi µL$^{-1}$). The tube was briefly centrifuged and 1 µL of DNA polymerase I large fragment (Klenow) was added and mixed gently. The reaction was incubated at room temperature for 4 hours. The probe was purified by passing the mixture through G-50 spin column (1000 g x 3 minutes). The $Cah6$ probe had a specific activity greater than 2 x 10$^8$ cpm µg$^{-1}$ DNA.
DNA PREPARATION, SEQUENCING AND HOMOLOGY ANALYSES

Total DNA was isolated from wild type D66 cells grown as patches on TAP plates according to Newman et al. (1990). Briefly, the cells were resuspended in disruption buffer containing SDS and then the nucleic acids were extracted using phenol/chloroform. The aqueous phase was extracted a second time with choloform. The nucleic acids were then precipitated with an equal volume of ethanol and washed twice with 70% ethanol.

Plasmid and cosmid DNA was purified using a combination of the standard ethanol precipitation method (Sambrook et al., 1989) followed by the purification method using the spin columns from a commercial kit (QIAGEN, Chatsworth, California). cDNA and genomic PCR products were purified from the 0.8% agarose gels. Amplified bands were cut from the gel and were treated with 6 M NaI at 55°C to melt the gel piece. DNA was purified from the liquefied gel using the mini spin columns from the commercial kit mentioned above. DNA molecular size standards were 2-log and 1 kb DNA (NEB, Beverly, Massachussets). For DNA concentration determination, HindIII digest of λ DNA was used as a standard (NEB, Beverly, Massachussets).

DNA was sequenced using ABI dye terminators (Perkin Elmer Applied Biosystems, Foster City, California); for some PCR fragments and cosmids enriched in the GC content, the use of dGTP-BigDye generated better sequences than dITP-BigDye). For plasmid clones greater than 6 kb, 800 ng of DNA was used per sequencing reaction. One-two µg of DNA was used to sequence cosmids. Ten and 40 ng of DNA were used to sequence PCR products (100-500 bp) and PCR products (500-1000 bp), respectively. The sequencing reaction contained 3.2 pMol of primers and 2 µL of Big Dye. The following PCR profile was used: 30 cycles at 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Homology searches (against Chlamydomonas EST and the full database) were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997). Exon/intron splice sites and open
reading frames were identified manually (Silflow, 1998) as well as by using GreenGenie (Kulp et al., 1996). Signal peptide analysis and molecular weight and pI calculations were determined with different protein prediction programs like CHLOR P, TARGET P and SORT P which had hyperlinks in the ExPasy server (http://ca.expasy.org/tools/#translate).

**cDNA LIBRARY PREPARATION AND AMPLIFICATION OF CAH6 AND GCLP1**

A cDNA core library (amplified bacteriophage libraries) was obtained from the *Chlamydomonas* Genetics Center at Duke University, Durham. This core library was made from cDNAs prepared from CC-1690 cells grown to mid-log phase in TAP (acetate-containing) medium in the light, TAP medium in the dark, HS (minimal) medium in ambient levels of CO₂ (0.035% CO₂) and HS medium bubbled with 5% CO₂. cDNAs were cloned into the lambda Zap II (Stratagene, La Jolla, California) in the EcoRI (5’) and XhoRI (3’) sites. The lambda ZAP II vector is designed to allow simple, efficient, *in vivo* excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. *In vivo* excision, involving the ExAssist interference-resistant helper phage along with the SOLR strain of *E. coli*, was used. The ExAssist helper phage contains an amber mutation that prevents the replication of the phage genome in a nonsuppressing *E. coli* strain like SOLR. This allows efficient excision of the pBluescript phagemid from the Lambda ZAP II vector while eliminating the problems associated with helper phage co-infection.

Overnight cultures (50 mL) of XL1 Blue MRF’ or SOLR cells were grown in LB broth at 30°C. Harvested cells were resuspended in 25 mL of 10 mM MgSO₄. The concentration of cells in 10 mM MgSO₄ was adjusted to a concentration of 1 x 10⁸ cells mL⁻¹. The amplified lambda bacteriophage library was mixed with the XL1 Blue cells in the ratio of 1:10. This was done to excise 10 to 100 fold more lambda phage than the size of the primary library to ensure statistical
representation of the excised clone. ExAssist helper phage was added at a 10:1 helper phage to cells ratio to ensure that every cell was co-infected with the lambda phage and the helper phage. The mixture was allowed to incubate at 37°C for 15 minutes to allow the phage to attach to the cells. Twenty mL of fresh LB broth were added to the mixture and allowed to incubate for 2-3.5 hours at 37°C with shaking. The mixture was then heated to lyse the phage particles and the cells. The lysed mixture was spun at 1000 g for 10 minutes to pellet the cell debris and the supernatant containing the excised phagemid was collected. The titer of the excised phagemid in the supernatant was determined and was used to calculate the number of colonies that were needed for the statistical representation of the excised clones. Colonies from all the culture plates were pooled by mass scraping and resuspension in 3 mL of LB. Plasmids were purified from these cells using the spin column method (QIAGEN, Chatsworth, California).

Cah6 primers F4 and R5 (Appendix 1) when used on the cDNA core library yielded a PCR product of 2452 bp. This product codes for the full length Cah6 protein. Gclp1 primers M1F and M1R yielded a PCR product of 1170 bp which codes for the full length Gclp1 protein.

SCREENING OF THE COSMID LIBRARY

To obtain wild-type genomic clones of the Cah6 and Gclp1 genes, a PCR-based screen of an indexed cosmid library was used. An indexed cosmid library was constructed using a cosmid library from Saul Purton, University of London (Purton and Rochaix, 1994). Briefly, 7680 different E. coli lines carrying single cosmids were grown in LB media on 80 different 96-well microtiter plates. Using this indexed library, 80 pools of cells, each containing 96 single cosmids, were generated. DNA from each pool, obtained by common alkaline lysis procedures (Sambrook et al., 1989), was used to create 10 superpools (each containing about 768 single cosmids) that were suitable for PCR. Using the sequence information obtained from the EST database of C. reinhardtii, primer sets were designed and used to screen the superpools. Cah6
primers F4 and R4 (Appendix 1) were used for PCR. These primers amplified a $Cah6$ product of 1.8 kb. Once a plate carrying the correct cosmid was identified, a new set of pools was generated (12 pools, each containing 8 single cosmids). Finally, a new PCR reaction was performed with the individual cosmids from the positive pool described above. Using this protocol, after 4 rounds of PCR, two cosmids containing the $Cah6$ gene, designated 72-E-6 and 29-D-12, were isolated from the cosmid library. PCR using primers F4 and R5 yielded a product of 2879 bp when used on the isolated cosmid clones. This PCR product is just six base pairs short from being the full length genomic sequence of $Cah6$.

The $Gclp1$ PCR primers 1F and 2B (Appendix 3) were used on genomic DNA to generate a product of 745 bp. This PCR product was used to make a probe to screen the indexed cosmid library. After three sequential rounds of screening, two cosmid clones namely 70-C-3 and 70-G-8 were isolated. Primers M1F and M1R were used on the two isolated cosmid clones to yield a PCR product of 2249 bp. The sequences of the $Cah6$ and $Gclp1$ primers and their alignments on the corresponding genomic maps are given in Appendix 1 and Appendix 3, respectively.

**PRODUCTION OF OVEREXPRESSION CONSTRUCTS**

$Cah3$, $Cah6$ and $Gclp1$ were cloned into the pMal-c2x overexpression vector (NEB, Beverly, MA) downstream from the $MalE$ gene which encodes maltose-binding protein (MBP). The vector (6648 bp) has an exact deletion of the $MalE$ signal sequence (bases 1531-1605) resulting in the cytoplasmic expression of the fusion protein. The vector contains the inducible Ptac promoter, which is a hybrid of Trp promoter and LacUV5 promoter. Ptac is positioned to transcribe a $MalE-LacZ\alpha$ gene fusion (Fig 2.1). The $LacI^q$ gene codes for the Lac repressor and has a promoter mutation which increases intracellular concentration of LacI repressor. It turns off transcription from Ptac until IPTG (Isopropyl-β, D-thiogalactopyranoside) is added (Fig. 2.1).
The polylinker cloning region provides restriction endonuclease sites to insert the gene of interest, fusing it to the MalE gene (Fig 2.2). Insertion of the desired gene into this site interrupts the LacZa ORF (open reading frame) allowing a blue-white selection on LB + Amp + X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) + IPTG plates. The vector also has Amp’ gene which codes for the β-lactamase gene that confers ampicillin resistance. This allows additional screening of transformants on LB + Amp plates. The fusion protein can be purified by one-step affinity chromatography using an amylose column. The vector has a spacer sequence coding for 10 asparagine residues between the MalE sequence and the polylinker sequence (Fig. 2.2). This allows insulation of MBP from the protein of interest, increasing the chances that a particular fusion protein will bind tightly to the amylose resin during purification. The vector also includes a sequence coding for the recognition site of a protease, Factor Xa, which allows the desired protein to be cleaved from MBP after purification.

The Cah3 cDNA sequence coding for the mature Cah3 protein was cloned into the pMal-c2x overexpression vector. "A" and "B" are the 5´ and 3´ end PCR primers, respectively used for amplification of Cah3 cDNA (790 bp). The primer A has the sequence 5´- AAG GGA TCC ATC GAA GGG CGC GCA GCT TGG ACC TAT TAT GGC GAA -3´ and the primer B has the sequence 5´- GTA AAG CTT CCC CAC CGT GGG CCA AAC -3’. The primer A has a Bam HI and a Factor Xa site incorporated at the 5´ end. Primer B has a HindIII site incorporated at the 5’end. The vector was double digested with HindIII and BamHI and ligated to the 790 bp Cah3 PCR product. This recombinant construct had two different Factor Xa cutting sites and was provided by Dr. Göran Samuelsson, Umeå University, Sweden. Two different pMal-Cah6 recombinant expression constructs were designed. One construct contained the cDNA sequence coding for the full length Cah6 protein and the other contained the cDNA sequence that would code for the putative mature Cah6 protein. SP2 + R4H primers and MP + R4H primers were
RECOMBINANT pMAL CONSTRUCT

Figure 2.1 A diagram showing some features of the pMal expression vector and the translation of the recombinant construct. $LacI^q$ stands for an allele that has a promoter mutation which increases intracellular concentration of LacI repressor, Ptac is a hybrid of Trp promoter and LacUV5 promoter, PCS stands for the polylinker cloning site, $GeneX$ is any gene cloned into the vector, $LacZa$ stands for the β-galactosidase gene and $Amp^r$ stands for the β-lactamase gene.
Figure 2.2 Partial sequence of the poly linker cloning site of pMal-c2x. The Factor Xa recognition amino acid sequence is shown in bold red. The Factor Xa cleavage site is denoted by a black line. *MalE* codes for the maltose binding protein (MBP) and *LacZα* codes for the β-galactosidase α-fragment. Restriction enzyme sites in the poly linker cloning region are shown on top.
used to amplify the cDNA coding for the full length and mature Cah6 protein, respectively. SP2 + R4H and MP + R4H primers yielded a PCR product of 1.1kb and 979 bp, respectively.

Like MBP-Cah6 constructs, two different MBP-Gclp1 expression constructs were made. S1 + M1RH primers and S3 + MIRH primers (Appendix 3) were used to amplify the cDNA coding for the full length and putative mature Gclp1 protein, respectively. S1 + M1RH and S3 + M1RH primers amplified a 1067 bp and 944 bp product, respectively.

A high fidelity DNA polymerase (Platinum Pfx DNA polymerase from Invitrogen, Carlsbad, California) was used for all PCRs. This was used to prevent any missense/null mutation in the protein sequence during PCR and generated blunt ended amplified products. The vector was double digested with the XmnI and HindIII. Amplified cDNAs were purified from the DNA gel using QIAGEN spin columns and were digested with HindIII. Ligation of the insert to the overexpression vector pMal-c2x vector was performed following the protocol in the New England BioLab technical manual.

Transformations of DH5α cells were performed following the protocol in Sambrook et al. (1989). Although transformants were isolated by screening both on LB + Amp (100 µg mL⁻¹) and LB + Amp (100 µg mL⁻¹) + IPTG (Isopropyl-β, D-thiogalactopyranoside; 1 mM) + X-Gal (80 µg mL⁻¹) plates, they were picked only from LB + Amp plates. Because of the strength of the Ptac promoter, transformants taken from a plate containing IPTG can contain mutant plasmids that have either, 1) lost part or all of the fusion gene, or 2) no longer express the protein at high levels.

Ampicillin (50 mg/mL) and IPTG (200 mM) stocks used for the experiment were filter sterilized. In-frame insertion of Cah6 and Gclp1 with the sequence of MBP in the recombinant clone was verified by double restriction enzyme digestion analyses with SaeI and HindIII and DNA sequencing.
OVEREXPRESSION AND PURIFICATION OF MBP FUSION PROTEINS

Selected clones of Cah3, Cah6 and Gclp1 were grown at 37°C in 2 L LB + glucose (0.2%) + Amp (100 µg mL⁻¹) cultures on a rotary shaker. Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the E. coli host, one of which codes for amylase which can degrade the amylose on the affinity resin that is used for purification. The cells were induced for 2 hours with 1 mM IPTG at 37°C when the culture OD₆₀₀ was between 0.6 - 0.7. Both induced and uninduced cells were harvested and resuspended in column buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid) with or without 10 mM 2-mercaptoethanol] and ruptured in a prechilled French pressure cell. Equal amounts of protein samples of ruptured induced and uninduced cells were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis to verify the overexpression of the recombinant protein.

The fusion protein was purified by one-step affinity chromatography using amylose resin (Fig. 2.3). Amylose resin (1 mL of amylose resin binds 3 mg of the recombinant protein) was mixed with the crude ruptured cell extract on a shaker at room temperature for three hours at room temperature and poured into a 2.5 cm x 10 cm column to perform batch purification. The column was washed with 6 L of column buffer to remove other proteins. At the final step, fusion proteins were eluted from the column by column buffer containing 10 mM maltose. Purified recombinant fusion proteins were further concentrated by a passage through the 100 kDa centric columns (Amicon, Billerica, Massachusetts). The recombinant proteins were recovered from the membrane of the filter in the centric columns.

Recombinant proteins were cleaved from the maltose binding protein (MBP) by digestion with the protease Factor Xa (NEB). Fifty µg of the recombinant protein were digested by 1 µg of Factor Xa enzyme in the Factor Xa digestion buffer [20 mM Tris-HCl, 100 mM NaCl, and 2 mM
Figure 2.3 A schematic diagram showing the amylose column purification of the recombinant fusion protein.
CaCl₂ (pH 8.0)] at 23°C for 4-6 hours. Purification and Factor Xa digestion of the recombinant protein was verified by performing 12% SDS-PAGE. SDS-PAGE was performed using pre-stained low molecular weight markers as protein standards (BioRad, Hercules, California).

GENERATION OF POLYCLONAL Cah3 AND Cah6 PRIMARY ANTIBODIES

Factor Xa digested purified recombinant proteins were separated on a 12% gel by SDS-PAGE at 15 mAmp for 18-20 hours. The 29 kDa Cah3 and 31 kDa Cah6 protein bands were excised carefully from the polyacrylamide gel. The gel sections were shipped to Strategic Dissolutions, Ramona, California, for production of antibody. Antibodies were raised against the Cah3 and Cah6 proteins by a standard 70-day protocol using two pathogen free rabbits. Approximately 1.6 mg of the protein was used to raise the antibody.

CARBONIC ANHYDRASE ASSAYS AND OTHER BIOCHEMICAL EXPERIMENTS

CA activity was assayed electrometrically using a modification of the Wilbur-Anderson method (Wilbur and Anderson, 1948). The samples were assayed at 4°C by adding 50 to 200 µL of the test sample to 3.5 mL of 20 mM EPPS (4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid; pH 8.0). The reaction was initiated by addition of 1.5 mL of ice cold CO₂ saturated water. The time required for the pH drop from 7.7 to 6.3 was measured. The activity of the test sample was calculated using the equation: \( WAU = \frac{t}{t_0} - 1 \) where \( t \) is the time required for the pH change when the test sample is present and \( t_0 \) is the time required for the pH change when buffer is substituted for the test sample. Bovine CAII (Sigma, St. Louis, Missouri) was used as a positive control. Both Factor Xa cut and uncut purified Cah6, Gclp1 and Cah3 fusion proteins were used for activity assays. Activity assays were also done using protein extracts from induced cells of the γ-CA (Cam) clone of Methanosarcina thermophila and purified Cam, which were kindly provided by Dr. J.G. Ferry, Pennsylvania State University, University Park, Pennsylvania. The effect of temperature on the activities of recombinant Cah6 and Cah3
proteins were studied. The temperatures used for this study were 0°C, 4°C, 25°C, 37°C, 45°C and 50°C. Recombinant Cah6 and Cah3 were incubated for 15 minutes at the indicated temperatures and cooled on ice. CA activity was measured at 4°C by the Wilbur-Anderson method. Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment.

The effects of the sulfonamide inhibitors ethoxyzolamide and acetazolamide and the anions azide and cyanide on CA activities of recombinant Cah6 and Cah3 were studied. The I<sub>50</sub> value corresponds to the concentration giving 50% inhibition. I<sub>50</sub> was determined by plotting the percentage of inhibition vs. the concentration of the inhibitor. Sodium salts of azide and potassium salts of cyanide were used. The effects of the thiol reducing agents like, cysteine, dithiothreitol and 2-mercaptoethanol, on CA activities of recombinant Cah6 and Cah3 were also studied. The recombinant proteins were purified without any thiol reducing agents in the buffer and the specific activities were measured. These specific activities in the oxidized state are denoted as 100% activities (ox-CA). To measure the CA activities in the reduced state, purified recombinant Cah3 and Cah6 enzymes were incubated for 30 minutes at room temperature with 10 mM 2-mercaptoethanol (Mer), 10 mM cysteine (Cys) or 10 mM dithiothreitol (DTT), and then the CA specific activities were measured. All the inhibitors and thiol reducing agents were purchased from Sigma.

**ELECTROPHORESIS AND IMMUNOBLOTTING**

For protein analyses and Western blots, cells were harvested, washed twice with fresh medium and resuspended in TEN buffer (10 mM Tris-HCl, 10 mM EDTA and 150 mM NaCl; pH 7.5). Proteins were separated on 12% and 15% polyacrylamide gels (0.8% bisacrylamide) as described previously (Laemmli, 1970). SDS-PAGE was performed using prestained low molecular weight markers as protein standards (BioRad, Hercules, CA). Immunoblotting was
performed as described in the protocol from BioRad Laboratories (Hercules, California). Cah6 primary antibody was used to probe proteins from high and low CO$_2$ adapted D66 and six different Cah6 RNAi mutant cells as well as the factor Xa cut and uncut purified MBP-Cah6 fusion proteins. The Cah6 antibody was diluted in the ratio of 1:1000 before being used as a probe.

Both the “new” and the “old” Cah3 primary antibodies were used to probe proteins from air adapted 137$, cia3, cia3 transformant 9A and ca-1 cells and also the factor Xa cut and uncut purified MBP-Cah3 fusion proteins. The “old” Cah3 primary antibody was kindly provided by Dr. Göran Samuelsson, Umeå University, Sweden. The “new” and the “old” Cah3 primary antibodies were diluted in the ratio of 1:1000 before being used as a probe. The primary antibodies were diluted with the antibody buffer (Tris-buffered saline + 0.005% Tween 20 + 1% bovine serum albumin, pH 7.4) before being used as probes. The antibody raised against the purified $\gamma$-CA from $M$. thermophila was kindly provided by Dr. J.G. Ferry, The Pennsylvania State University, University Park, Pennsylvania. The secondary antibody used for Western blotting was conjugated to the enzyme horseradish peroxidase (BioRad, Hercules, California) and diluted at a ratio of 1:3000 with the antibody buffer. Western blots were developed following the protocol from BioRad using a mixture of the horse radish peroxidase color development reagent (BioRad) in ice cold 100% methanol (20 mL), Tris-buffered saline (80 mL; pH 7.4) and 30% H$_2$O$_2$ (60 $\mu$L).

**IMMUNOLOCALIZATION STUDIES USING ELECTRON MICROSCOPY**

Air adapted D66 cells were fixed in a mixture of 1% OsO$_4$, 2% formaldehyde and 0.5% glutaraldehyde in a 1:1 ratio for 15 minutes. The sample was then fixed for an additional 15 minutes in 1% OsO$_4$, 2% formaldehyde, 0.5% glutaraldehyde and 0.1 mM sodium cacodylate buffer. Materials were rinsed with distilled water and stained with 0.5 % uranyl acetate for 30
minutes. After this, excess stain was rinsed and the samples were dehydrated in ethyl alcohol series. Samples were then infiltrated and embedded in L.R. White resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania). Embedded *Chlamydomonas* tissues were sectioned with a Dupont Sorvall microtome and the sections were 70 µm thick. The immunocytochemical procedure was similar to the method of Borkhsenious *et al.* (1998) with some modifications. Sections were pretreated with 2.5% sodium-meta periodate (Sigma, St. Louis, Missouri) for 10 minutes to remove residual glutaraldehyde, rinsed in distilled water and blocked twice for 20 minutes each with 2% BSA and 0.1% Tween 20 in PBS (*Phosphate salme buffer*, Sigma).

The sections were then incubated for 90 minutes with diluted primary antibody (1:10 dilutions of the Cah6 primary antibody) or with the preimmune serum diluted similarly (used as a negative control). Sections were then washed with 0.5% Tween 20 six times for a total of 30 minutes and blocked again with 2% BSA (*Bovine serum albumin*) for 10 minutes. The grids were transferred to 1:50 dilution of Protein A (Sigma) conjugated to 20 nm colloidal gold particles. Protein A was diluted with 1% BSA and 0.1% Tween 20 in PBS for 1 hour. Sections were washed with PBS four times; each rinse was for 5 minutes. Finally the sections were rinsed with distilled water, dried, viewed and photographed by transmission electron microscopy. Sectioning of *Chlamydomonas* cells, immunolocalization and transmission electron microscopy works were done by Ying Xiang (Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana).

**CLONING TO GENERATE CAH6 RNAi MUTANTS**

RNA interference (RNAi) was used in an attempt to generate *Cah6* RNAi mutants. The silencing of a gene triggered by the presence of aberrant RNA of that gene in the host cell is generally termed as “RNA interference”. This abnormal RNA could be a double stranded RNA, a shortened RNA that lacks its “cap” or “tail” or a conventional RNA that is present in unusually
large quantities. All of these may indicate to a cell that a virus is on the attack. The cell responds by degrading these abnormal RNAs. The degradation of these abnormal RNAs is usually accompanied by the suppression of the specific gene in the host cell coding for these abnormal RNAs. Artificially, a RNAi construct of a gene that one wants to silence can be produced by cloning two reverse complementary sequences of the same gene in a vector (Fig. 2.4). This vector can be used to transform a wild type cell. Upon transcription, the RNAi construct leads to the production of double stranded RNA molecules specific to the gene that one wants to underexpress (Fig. 2.4). In the cell, these double stranded RNA molecules are amplified by RNA-dependent RNA polymerase and cleaved into 20-25 bp small fragments. These cleaved double stranded RNA fragments are denatured. The antisense strands from the denatured double stranded RNA molecules bind to the host target mRNA specific to the gene and degrades it. This leads to the lack of expression or underexpression of the target protein which in turn generates the RNAi “mutants” of the target gene. These mutants can be screened under appropriate conditions to study the functional role of the target gene (Fig. 2.4).

Cah6 was cloned into the pSL72 vector which is 5.5 kb long to generate RNAi mutants. The pSL72 vector was kindly provided by Dr. S. D. Lemaire (University of Paris, Orsay, France). The pSL72 vector contains the *AphVIII* and *Amp*\(^r\) genes which code for an aminoglycoside phosphotransferase (from *Streptomyces rimosus*) and \(\beta\)-lactamase, respectively. *AphVIII* and *Amp*\(^r\) confer paromomycin and ampicillin resistance, respectively. *Amp*\(^r\) can be a selective marker in bacteria. *AphVIII* can be a selective marker in *C. reinhardtii* as paromomycin inhibits eukaryotic translation. The *AphVIII* gene has a constitutive PsaD promoter and terminator from *C. reinhardtii*. It also has an intron sequence which is the second intron of the cytochrome \(c_6\) gene of *C. reinhardtii* (Fig. 2.5). Two different RNAi constructs (5′ and 3′ end constructs) of Cah6 were generated by cloning reverse complementary sequences of
Figure 2.4  A schematic figure showing the underexpression of the protein by RNA interference. A. A wild type cell expressing gene X. B. The wild type cell shown in (A) is transformed by the RNAi construct of gene X.
Figure 2.5  A schematic figure of the pSL72 vector. The vector is 5495 bp long. *AphVIII* and *Amp* genes code for an aminoglycoside phosphotransferase (from *Streptomyces rimosus*) and β-lactamase respectively. *AphVIII* and *Amp* confer paromomycin and ampicillin resistance, respectively. The PsaD promoter and terminator are from *C. reinhardtii*. The intron sequence is the second intron of the cytochrome *c*₆ gene of *C. reinhardtii*. The restriction enzyme sites that were used for cloning are given on the top of the construct by black lines.
Cah6 in the pSL72 vector (Fig 2.6). The 5’ end of the construct contained both genomic and cDNA sequences of a selected 5’ end region of the Cah6 gene that were reverse complementary to each other. RNAi primers X1 + X2 (Appendix 2) were designed to amplify a product of 702 bp of Cah6 from the cosmid 72-E-6. The genomic product had two introns of Cah6. X3 + X4 primers (Appendix 2) were used to amplify a product of 457 bp of Cah6 from the cDNA core library which is reverse complementary to X1 + X2 PCR product. The 3’ end construct had selected reverse complementary 3’ UTR cDNA sequences of Cah6 (Fig 2.6). Sequences of these RNAi primers and their alignments on the genomic or cDNA map are shown in Appendix 2. The primers X5 + X6 and X7 + X8 (Appendix 2) were designed to amplify the two 609 bp reverse complementary cDNA PCR products that were used to generate the 3’ end construct. The 5’ and 3’ end regions of Cah6 were selected based on comparative DNA sequence analyses of the mitochondrial β-CAs (Ca1 and Ca2) and Cah6 genes.

Reverse complementary sequences were cloned in two steps. In the first round of cloning, PCR products generated by X1 + X2 and X5 + X6 primers were digested by EcoRI and SmaI. The vector was double cut with the EcoRI and EcoRV. PCR products (X1 + X2 and X5 + X6) were ligated separately before the 5’ end of the cytochrome c6 intron in the pSL72 vector to generate 5’ (clone # 6) and 3’ (clone # 3) end preliminary RNAi constructs, respectively (Fig. 2.6). Cah6 transformants containing part of the Cah6 gene were screened on LB + Amp (100 µg mL⁻¹) plates and positive clones were verified by restriction digestion with AvrII and EcoRI. In the second cloning step, PCR products generated by X3 + X4 and X7 + X8 primers were digested with Bam HI (at the 5’ end) and XbaI (at the 3’ end) and separately ligated to the 5’ (clone # 6) and 3’ (clone # 3) end preliminary RNAi constructs, respectively obtained in the first round of cloning. The Cah6 DNA fragments (X3 + X4 and X7 + X8) in the second round of cloning, were cloned after the 3’ end of the cytochrome c6 intron in pSL72 vector (Fig. 2.6).
Transformants were screened on LB + Amp plates as before. Double restriction enzyme digestions of the selected clones with various combinations of restriction endonucleases were done (EcoRI and AvrII, BamHI and XbaI, Bam HI and AvrII and AvrII and XbaI). These restriction digestion results were verified by DNA sequencing. This led to the identification of the 5′ (clone # 11A) RNAi and 3′ (clone # 10B) RNAi clones. Bacterial transformation was done according to the protocol in Sambrook et al., (1989) and the ligations were done using T4 DNA ligase from NEB, following their protocol.

TRANSFORMATION OF C. reinhardtii AND SCREENING OF RNAi MUTANTS

The strain D66 (nit2-, cw15, mt+) was used for transformation. Culture conditions were similar to those used previously (Rawat and Moroney, 1991). D66 cells were first grown on 100 mL of Tris-Acetate-Phosphate (TAP) medium (Sueoka, 1960) for 2 days and then transferred to 1 L of TAP medium 24 hours before the experiment. Cells were harvested and resuspended at a density of 2 x 10⁸ cells mL⁻¹. For electroporation, 1 µg of circular or linearized DNA of 5′ and 3′ Cah6 RNAi constructs were added to 300 µL of the resuspended cells in electroporation cuvettes (0.4 cm gap width; BioRad Laboratories, Hercules, California). DNA was linearized by digestion with KpnI. These mixtures were placed on ice for 15 minutes. A BioRad electroporator Gene pulser II at a capacitance of 25 µF, without a shunt resistor, a voltage set at 2000 V cm⁻¹ and a pulse time between 9.4 and 10 ms, was used for electroporation. After electroporation, the cells were allowed to recover overnight in 10 mL of TAP + 60 mM sucrose medium in the dark. The next morning, the cells were harvested and resuspended in 4 mL of TAP + 0.5% agar. Cells were plated onto TAP + paromomycin (7.5 µg mL⁻¹) plates and were allowed to grow under very low-light conditions. Transformants containing the RNAi constructs had the paromomycin resistance gene and grew on TAP + paromomycin plates. Three hundred 5′ RNAi and 600 hundred 3′ RNAi paromomycin resistant colonies were transferred to
Figure 2.6 RNAi constructs of Cah6. A. 3’end Cah6 RNAi construct. B. 5’end Cah6 RNAi construct. The reverse complementary fragments of Cah6 were cloned in the pSL72 vector. Restriction enzymes used to cut the vector and the Cah6 DNA fragments are shown by straight black lines. AphVIII and Amp’ code for paromomycin and ampicillin resistance, respectively. PsaD promoter and terminator are from C. reinhardtii. PsaD promoter is a constitutive promoter. The intron represented by the green box is the second intron of the cytochrome c6 gene of C. reinhardtii. Two pink boxes in the 5’ end Cah6 RNAi construct represent the two introns in the 5’ end of Cah6 gene.
fresh TAP-paromomycin plates. After one week, 5-6 colonies from the 5’ RNAi and 3’ RNAi plates were randomly picked and screened under high and low CO₂ conditions on MIN plates for the presence of Cah6 protein using Western blotting.

OTHER ANALYTICAL METHODS

The CO₂ concentration in the growth chambers was measured using an infrared gas analyzer (The Analytical Development Co. Ltd, Hoddesdon, England) which reads at an accuracy of ± 2%. The CO₂ concentration was checked at least once, everyday, while the alga was growing in the high and low CO₂ growth chambers. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Chlorophyll concentration was determined spectrophotometrically (Arnon, 1949) using the equations of Holden (1976). 100% methanol was used as a solvent for extraction of chlorophyll. Cell density values were determined by direct counting in a hemocytometer chamber.
CHAPTER 3

OVEREXPRESSION, PURIFICATION AND PARTIAL CHARACTERIZATION OF THE α-CA PROTEIN Cah3

INTRODUCTION

Of the three CA families, the α-CA is the most studied and has a very wide distribution. Although α-CAs have long been known to occur in animals (Meldrum and Roughton, 1933), they have only recently been identified in algae (Fukuzawa et al., 1990; Fujiwara et al., 1990), the higher plant Arabidopsis thaliana (Moroney et al., 2001) as well as in eubacteria (Soltes-Rak et al., 1997; Elleby et al., 2001; Chirica et al. 2001) and viruses (Niles et al., 1986; Strayer and Jerng, 1992). Most α-CAs are active as monomers of about 30 kDa with three histidines coordinating the zinc atom (Moroney et al. 2001). All known α-CAs are highly susceptible to inhibition by sulfonamide compounds (Moroney et al., 1985). More information about α-CAs may be found in Chapter 1.

Three α-CAs have been identified to date in the green alga C. reinhardtii. In C. reinhardtii, two of these α-CAs (Cah1 and Cah2) are compartmentalized in the periplasm and one (Cah3) occurs in the lumen of the thylakoid. Cah1 and Cah2 were the first α-CA genes cloned from a photosynthetic organism (Fukuzawa et al., 1990; Fujiwara et al., 1990). These two genes encode very similar proteins although they are regulated differently. Cah1 is expressed under low CO2 conditions but not under high CO2 conditions while Cah2 is poorly expressed under low CO2 and slightly upregulated under high CO2 conditions. In addition, the expression of Cah2 under high CO2 appears low compared to the expression of Cah1 under low CO2 conditions (Fujiwara et al., 1990). A HCR mutant, cia5, fails to produce the low CO2-induced CA and any of the proteins associated with the CCM. These results seem to indicate
that Cah1 is required for the functional operation of CCM in *C. reinhardtii* (Moroney *et al.*, 1985). Recent studies have cast some doubt on the role of Cah1 since a *Chlamydomonas* mutant defective in the *Cah1* gene and protein appears to perform as well as wild type cells at limiting *C*<sub>i</sub> conditions (Van and Spalding, 1999). Thus at this point the evidence for the role of Cah1 in the CCM is not very clear. The function of Cah2 is also obscure (Moroney *et al.*, 2001).

Cah3 from *C. reinhardtii* was discovered in 1995 (Karlsson *et al.*, 1995). This α-CA is constitutively expressed and is slightly upregulated under low CO<sub>2</sub> conditions. A *C. reinhardtii* mutant, *cia3*, which has two point mutations in the region coding for the transit peptide of the Cah3 protein, grows poorly under low CO<sub>2</sub> but grows well under high CO<sub>2</sub>. *cia3* and *ca-1* (another HCR mutant), were complemented by the wild type Cah3 gene (Karlsson *et al.*, 1998; Funke *et al.*, 1997) and the complemented cells grew well under low CO<sub>2</sub>. This shows that Cah3 is required for the growth of *C. reinhardtii* in air levels of CO<sub>2</sub> (Funke *et al.*, 1997). This also shows that the mutation in both mutants affects the same gene. The sequencing of the genomic region of *ca-1* revealed a point mutation in the 5´ end of the ORF (open reading frame) creating a stop codon. Translation of the Cah3 mRNA in *ca-1* led to an incomplete and inactive polypeptide which was thirteen amino acids long. Karlsson *et al.* (1998) published the sequence of the *Cah3* cDNA. This group had cloned the Cah3 cDNA sequence coding for the mature Cah3 protein in the pMal-c2x overexpression vector. They had fused the Cah3 cDNA at the 3´ end of the *MalE* gene in this vector. *MalE* codes for the maltose binding protein (MBP). More information about the cloning and the MalE-*Cah3* recombinant construct has been presented in Chapter 2.

The *MalE-Cah3* recombinant construct and the Cah3 primary antibody raised against the purified uncut MBP-Cah3 protein were kindly provided for my research by Dr. Göran Samuelsson (Umeå University, Sweden). Here I report my studies of the Cah3 protein.
Although the Cah3 gene has been well characterized, there is no past report of the detection and measurement of CA activity of the purified protein. This is the first report of detection of significant in vitro CA activity in a purified Cah3 protein and partial characterization of this CA activity.

RESULTS

1. Overexpression of the MBP-Cah3

The original Cah3 antibody (“old”) was generated by Dr. Samuelsson’s laboratory (Umeå University, Sweden) against the purified MBP-Cah3 recombinant fusion protein (Karlsson et al., 1998). This “old” antibody did not cross react with Cah3 specifically. Hence Cah3 was overexpressed and purified primarily to raise an antibody against it for Western blotting and immunolocalization studies. E. coli cells harboring the recombinant MalE-Cah3 construct (Fig. 3.1) were induced with 1 mM IPTG for 2 hours at 37°C to overexpress the MBP-Cah3 fusion protein (Chapter 2). The overexpressed recombinant Cah3 protein comprised approximately 10% of the total E. coli cell protein (Fig. 3.2).

2. Purification and Activity Assays of the Recombinant Cah3

Overexpressed recombinant Cah3 protein was purified by affinity chromatography using amylose resin. The purified fusion protein was further concentrated by using 100 kDa cut-off centricon columns. At each step of purification of the recombinant Cah3 protein from crude E. coli cell sample, the CA activity in the sample was assayed in order to check the purity of the Cah3 sample (Table 3.1). The purified recombinant Cah3 had a specific activity of 300 WAU/mg (Wilbur and Anderson unit/mg). This calculation of the specific activity of Cah3 was based on the total amount of recombinant fusion protein in the sample. CA activity was not detected in either the cell extracts from uninduced E. coli cells harboring the recombinant MalE-Cah3 plasmid or E. coli cells containing only the pMal vector. The fusion protein was cleaved by
Figure 3.1  A schematic diagram of the recombinant MalE-Cah3 expression construct. A. schematic figure showing the alignment of primers used to amplify the Cah3 cDNA. A and B are 5’ and 3’ end PCR primers, respectively for amplification of the Cah3 cDNA. Primer B has a HindIII site incorporated at the 5’ end. Primer A has a BamHI and a Factor Xa site incorporated at the 5’ end. The numbers on the top denote the primer positions in base pairs. B. Part of the pMal vector showing the polylinker cloning site (PCS), β-galactosidase (LacZα), β-lactamase (Ampr) genes, BamHI and HindIII recognition sites and Factor Xa cleavage sites. *MalE* codes for the MBP. C. A schematic figure of the Cah3 recombinant construct.
Figure 3.2 A 12% SDS-polyacrylamide gel showing the uninduced and induced *Escherichia coli* cells expressing the recombinant MBP-Cah3 protein. Lane ST contains prestained molecular weight markers. Lane 1 contains 20 µg of proteins from uninduced *E. coli* cells. Lane 2 contains 20 µg of proteins from induced *E. coli* cells expressing chimeric MBP-Cah3 protein. Proteins were stained with Coomassie Blue after 12% electrophoresis.
Table 3.1 The purification of the chimeric MBP-Cah3 from 2 liters of *E. coli* culture

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (WAU)(^a)</th>
<th>Protein (mg)(^b)</th>
<th>Specific activity (WAU/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated cells</td>
<td>1000</td>
<td>8333</td>
<td>0.12</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Amylose column</td>
<td>800</td>
<td>5</td>
<td>160</td>
<td>80</td>
<td>1334</td>
</tr>
<tr>
<td>Centricon column</td>
<td>300</td>
<td>1</td>
<td>300</td>
<td>30</td>
<td>2500</td>
</tr>
</tbody>
</table>

\(^a\)one WAU = \(t_0-t\)/ \(t\) where \(t_0\) is the time for uncatalyzed reaction and \(t\) is the time for the enzyme catalyzed reaction; \(^b\)determined by Lowry’s protein assay (Lowry *et al.*, 1951)
the protease Factor Xa for four hours at 23°C to cleave Cah3 from the MBP. Purification and cleavage of the fusion protein was confirmed by SDS-PAGE (Fig. 3.3). The 29 kDa Cah3 protein band was excised from the gel to be used as a source antigen for production of the new polyclonal Cah3 primary antibody (“new”).

3. Western Blotting

The original Cah3 antibody (“old”) was generated by Dr. Samuelsson’s laboratory (Umeå University, Sweden) against the purified MBP-Cah3 recombinant fusion protein (Karlsson et al., 1998). Factor Xa-cleaved Cah3 and purified MBP-Cah3 fusion proteins were separated by 12% SDS-PAGE and probed with the “old” and the “new” Cah3 primary antibodies (Fig. 3.4 and Fig. 3.5). The “old” Cah3 antibody reacted equally well with the MBP and Cah3 (Fig. 3.4). The new Cah3 antibody reacted specifically with the Cah3 protein since it was separated from the MBP from the gel before raising the antibody (Fig. 3.5).

Proteins were extracted from cells (grown at air levels of CO₂) of different strains of C. reinhardtii, namely WT 137, ca-1 (cah3 mutant), cia3 (cah3 mutant) and 9A (a cia3 mutant transformed with the wild type Cah3 gene). These proteins were separated by 10% SDS-PAGE and probed with both the “new” and the “old” Cah3 primary antibody (Fig. 3.6). The “new” Cah3 antibody detected the Cah3 protein (29 kDa band) in the WT 137 and 9A cells but not in the two cah3 mutants namely ca-1 and cia3. Moreover, the 9A transformant cells possessed approximately twice the amount of Cah3 protein than that of the WT 137 (Fig. 3.6). The “old” Cah3 antibody picked up two distinct proteins having very similar molecular weights. The top and the bottom protein bands picked up by the “old” antibody are approximately 30 kDa and 29 kDa, respectively. The bottom protein band (29 kDa) matches the 29 kDa protein band detected by the “new” Cah3 antibody and is that of Cah3 protein. This 29 kDa Cah3 protein was missing in the ca-1 and cia3 mutants but present in WT137 and 9A transformant. This 30 kDa band was
Figure 3.3  A 12% SDS-polyacrylamide gel showing the purified uncut and the Factor Xa cut MBP-Cah3 protein. Lane ST represents the prestained molecular weight markers. Lane 1 contains 20 µg of uncut chimeric MBP-Cah3 protein. Lane 2 contains 20 µg of chimeric MBP-Cah3 cut by 1 µg of Factor Xa. Lane 3 contains 1 µg of Factor Xa. Proteins were stained with Coomassie Blue after 12% electrophoresis.
Figure 3.4 A Western blot probed by the “old” Cah3 antibody raised against the recombinant mature MBP-Cah3 protein. ST represents the low molecular weight prestained standards. Lane 1 contains 25 µg of purified MBP-Cah3 cut with 1 µg of Factor Xa. Lane 2 contains 25 µg of purified uncut MBP-Cah3. Lane 3 contains 1 µg of Factor Xa. The proteins were run on a 12% SDS-polyacrylamide gel followed by Western blotting.
Figure 3.5  A Western blot probed by the “new” Cah3 primary antibody.  Lane ST represents the prestained molecular weight markers.  Lane 1 contains 1 µg of Factor Xa.  Lane 2 contains 20 µg of purified chimeric MBP-Cah3 cut by 1 µg of Factor Xa.  Lane 3 contains 20 µg of purified uncut chimeric MBP-Cah3.
picked up by the “old” antibody when it was used to probe proteins extracted from spinach leaves (data not shown). The identity of the top 30 kDa band is unknown at this point. These two proteins were not resolved on a 12% or 15% gel as they migrated together on the gel (Fig. 3.7). A number of labs (Park et al., 1999; Villarejo et al., 2001; Villarejo et al. 2002; Karlsson et al., 1998) have used this “old” Cah3 antibody for research, and the mutant cia3 has been reported to have the Cah3 protein, when the “old” Cah3 antibody was used as a detecting tool (Karlsson et al., 1998). There is currently no published report stating that ca-1 has the protein.

4. Partial Characterization of the Cah3 Activity

Since there appears to be no significant difference between the catalytic activity of Factor Xa-cleaved MBP-Cah3 and the fusion protein, all the biochemical experiments were done with the purified MBP-Cah3 fusion protein. Since sulfonamide compounds and certain anions are known to inhibit CA activity (Johansson and Forsman, 1993), the effects of sulfonamide and anion inhibitors on the CA activity of recombinant Cah3 were studied (Table 3.2). Sulfonamides were more potent as inhibitors than monovalent anions (cyanide and azide). Ethoxyzolamide was a more effective inhibitor than acetazolamide. Azide was slightly more effective as an inhibitor than cyanide.

The effects of different temperatures on the Cah3 CA activity are shown in Fig. 3.8. The enzyme lost more than 50% of its activity above 43°C and became inactive at 50°C. The sensitivity of recombinant Cah3 to reduction was studied. Different SH-reducing agents were used to reduce Cah3 and observe the reduction effects on the CA activity of recombinant Cah3. Cah3 lost most of its CA activity upon exposure to SH-reducing agents. Purification of the enzyme with buffers lacking a SH-reducing agent yields an enzyme with high activity as shown in Fig. 3.9. The result seems to indicate that the enzyme is more active in the oxidized state than the reduced state.
Figure 3.6 Western blots probed by the “old” and “new” Cah3 primary antibodies when samples were separated by 10% SDS-PAGE. ca-1 and cia3 are two different cah3 mutants of C. reinhardtii. 9A is cia3 mutant transformed with the wild type Cah3 gene. WT 137 represents the parental wild type cell of cia3. ST represents the prestained molecular weight markers.
Figure 3.7 Western blots probed by the “old” Cah3 primary antibody when samples were separated by 15% SDS-PAGE. ca-1 and cia3 are two different cah3 mutants of C. reinhardtii. 9A is cia3 mutant transformed with the wild type Cah3 gene. WT 137 represents the parental wild type cell of cia3. ST represents the prestained molecular weight markers.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$I_{50}$ of bovine CAII (M)</th>
<th>$I_{50}$ of Cah3 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>$1.4 \times 10^{-8}$</td>
<td>$8 \times 10^{-9}$</td>
</tr>
<tr>
<td>Ethoxyzolamide</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$6 \times 10^{-9}$</td>
</tr>
<tr>
<td>Azide</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$32 \times 10^{-6}$</td>
</tr>
<tr>
<td>Cyanide</td>
<td>$4.9 \times 10^{-5}$</td>
<td>$59 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

Erythrocyte Bovine CAII was purchased from Sigma. The $I_{50}$ value corresponds to the concentration giving 50% inhibition. $I_{50}$ was determined by plotting percentage of inhibition vs. concentration of inhibitor. Sodium salts of azide and potassium salts of cyanide were used. The data shown for each treatment in the table are the averages ± SD of three different measurements.
**Figure 3.8 The thermostability of Cah3 activity.** Cah3 was incubated for 15 minutes at the indicated temperatures and cooled on ice. CA activity was measured at 4°C by the Wilbur-Anderson method (1948). Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment (300 WAU/mg). The data shown for each temperature treatment in the graph are the averages ± SD of three different measurements.
Figure 3.9 The effects of the SH-reducing agents on Cah3 activity. Cah3 was purified without any reducing agents in the buffer and the activity was measured. The specific activity (300 WAU/mg) in the oxidized state is denoted as 100% activity (ox-CA). Prior to the measurements of CA activity, purified recombinant Cah3 enzyme was incubated for 30 minutes at room temperature with: 10 mm 2-mercaptoethanol (Mer), 10 mM Cysteine (Cys) and 10 mM dithiothreitol (DTT). The data shown for each treatment in the graph are the averages ± SD of three different measurements.
DISCUSSION

This is the first report of the detection of CA activity in purified recombinant Cah3 and partial characterization of this CA activity. Cah3 is an \( \alpha \)-CA located in the thylakoid lumen. This \( \alpha \)-CA is constitutively expressed and slightly upregulated under low CO\(_2\) (Karlsson et al., 1998). Cah3 has 30%-40% amino acid identity with other known \( \alpha \)-CAs and is most similar to the \( \alpha \)-CA from \textit{Neisseria gonorrhoeae} [(40.6% identity); Karlsson et al., 1998]. \( \alpha \)-CAs are known to have a broad range of specific activity ranging from 5 WAU/mg (human CAIII) to 2000 WAU/mg (human CAII). The specific activity of recombinant Cah3 enzyme is around 300 WAU/mg, comparable to human CAI (an \( \alpha \)-CA) specific activity. The calculation of the specific activity in the present study was based on the total amount of recombinant fusion protein in the sample, and MBP and Cah3 have a molecular weight of 42 kDa and 29 kDa, respectively. Since the molecular size of MBP is 1.44 times greater than that of Cah3, the actual specific activity of Cah3 would be approximately 730 WAU/mg. The recombinant mature Cah3 was heterologously produced in \textit{E. coli}. It is known that proteins expressed in bacterial cells sometimes are not folded properly or mostly get aggregated in inclusion bodies. Significantly, less than 10% of the total purified Cah3 protein was in the soluble fraction and was active. This is evident from the extent of purification and specific activities (shown in Table 3.1), and the SDS-PAGE results (Fig. 3.2), respectively. Cah3 is targeted to the thylakoid lumen which has an acidic pH around 4. Cah3 might have an optimum pH around 4. CA activity assays were done at pH 8 as the Wilbur and Anderson CA activity assay method works best around pH 8.5-pH 7.4. This could have had an effect on the Cah3 CA activity.

Cah3 has an apparent molecular weight of 29 kDa on a SDS-polyacrylamide gel and a pI of \(~7.87\). The calculated molecular weight of mature Cah3 is around 26 kDa. It is not a membrane spanning protein as it can be released into the soluble fraction by washing the
thylakoids with 300 mM KCl (Karlsson et al., 1998). It was initially suggested that Cah3 speeds up the formation of CO₂ from HCO₃⁻ in the acidic lumen of thylakoids and that this CO₂ diffuses through the thylakoid membrane to the pyrenoid (Moroney and Mason, 1991; Badger and Price, 1994; Raven, 1997; Moroney and Somanchi, 1999). This model is based on the assumption that HCO₃⁻ is actively pumped into the lumen from the stroma and on the observation that in many algae, including *Chlamydomonas*, thylakoids penetrate the pyrenoid matrix in various patterns (Raven, 1997).

It has also been proposed that the activity of this CA stabilizes the manganese cluster of PSII and maintains the oxygen evolution complex (OEC) in a functionally active state (Villarejo et al., 2002). Recently, Hanson et al., (2003) have proposed that Cah3 mainly provides elevated levels of CO₂ to Rubisco under low CO₂ conditions and under high light, and might indirectly aid in the stabilization of PSII by preventing overacidification of the thylakoid lumen through rapid dehydration of HCO₃⁻ to CO₂ and H₂O.

Cah3 has three histidine residues that coordinate the zinc atom, and is highly susceptible to sulfonamide compounds like all other α-CAs. Recombinant Cah3 is more susceptible to acetazolamide, azide and cyanide and slightly less susceptible to ethoxyzolamide than bovine CAII. Membrane impermeant inhibitors of CAs like acetazolamide (AZ) inhibits CO₂ fixation and Ci accumulation in wild type *C. reinhardtii* cells (Moroney et al., 1985). Ethoxyzolamide (EZ) is a membrane permeable CA inhibitor. When physiological studies were performed using EZ on the air adapted wild type *Chlamydomonas* cells, the cells showed the same physiological properties as the air adapted cia3 cells (Spalding et al., 1983; Karlsson et al., 1998). These physiological traits include reduction in internal CA activity, a high photosynthetic CO₂ compensation point, oxygen sensitive photosynthesis, high rates of glycolate accumulation and excessive accumulation of HCO₃⁻ compared to the wild type cells. These observations suggest
that both the periplasmic and thylakoid α-CAs (Cah1 and Cah3) play an important role in CCM and photosynthesis.

Cah3 loses more than 50% of its activity at temperatures at 43°C and 100% of its CA activity at 50°C. It has an optimal activity around 33°C. Thus the optimal temperature for Cah3 activity falls within the range of the optimal growth temperature (12°C-35°C) of *C. reinhardtii*. The enzyme needs to be maintained in the oxidized state to retain its CA activity as exposure to thiol reducing agents reduces its CA activity significantly. This likely indicates that disulfide bond formation in Cah3 is essential for its CA activity. Cah3 has three cysteine residues in the mature protein. Site directed mutagenesis experiments can be done to specifically substitute these cysteines in Cah3 to study whether or not cysteines play a role in controlling the CA activity of Cah3. The cytoplasm of cells and the stroma of chloroplasts in general is expected to have a greater reducing environment than that inside the endoplasmic reticulum or thylakoid lumen (Personal communication Dr. Bob Buchanan, University of California, Berkeley, California). The redox environment in the thylakoid lumen can favor the oxidized form of the enzyme which is more active than the reduced form.

All the CA activity measurements and studies related to the partial characterization of the CA activity of Cah3 presented here are based on *in vitro* analyses of the recombinant Cah3 protein and need to be confirmed by *in vivo* analyses. *In vivo* activity measurement of Cah3 is a challenging task because of the presence of multiple isoforms of CAs from the three CA families in different cell compartments in *C. reinhardtii*.

Western blotting results showed that the “new” Cah3 antibody detects Cah3 more specifically than the “old” Cah3 primary antibody that was raised against the MBP-Cah3 fusion protein. The “old” Cah3 antibody cross reacts strongly with two different proteins that had similar molecular weights. These two proteins were not resolved on a 12% or 15% gel as they
migrated together on the gel (Fig. 3.7). Hence, with the “old” Cah3 antibody it was impossible to deduce if ca-1 and cia3 mutants lack the Cah3 protein, when protein samples were separated on a 12%-15% SDS-PAGE gel. cia3 has two point mutations in the region coding for the thylakoid targeting transit peptide of the Cah3 protein. These mutations lead to the mistargeting of the Cah3 protein. ca-1 has a stop codon leading to a truncated protein, only thirteen amino acids long. In ca-1, it is expected that such a highly truncated protein would be degraded. In cia3, the mistargeted protein could be either degraded or be present in the stroma of the chloroplast. Western blots with the “new” Cah3 antibody seem to indicate that the protein is missing in ca-1 and cia3. This result needs to be confirmed by the immunolocalization studies. Currently immunolocalization experiments are being done using the “new” Cah3 antibody to determine if the two cah3 mutants namely cia3 and ca-1, in reality, lack or underexpress the Cah3 protein.
CHAPTER 4

IDENTIFICATION OF A NEW β-CA GENE (Cah6) AND PARTIAL CHARACTERIZATION OF ITS PROTEIN PRODUCT

INTRODUCTION

β-CAs were the first carbonic anhydrases to be identified in photosynthetic organisms (Burnell et al., 1990; Fawcett et al., 1990). The distribution of β-CAs does not appear as widespread as the α-CAs. β-CAs have been found in higher plants, micro-algae, eubacteria (Hewett-Emmett and Tashian, 1996), archaebacteria (Smith and Ferry, 1999), the fungi Saccharomyces cerevisiae and S. pombe (Götz, et al., 1999), Caenorhabditis elegans (putative β-CA genes exist in the EST database), Drosophila melanogaster (putative β-CA genes exist in the EST database) but not in vertebrates. In C₃ plants, an abundant β-CA is localized to the chloroplast stroma and is highly active.

β-CAs have been found both in the cytoplasm (C₄ mesophyll cells) and chloroplast (C₃ plants) of higher plants and in the symbiotic green alga Coccomyxa (Hiltonen et al., 1998). In A. thaliana, cDNAs encoding cytoplasmic and chloroplastic forms of β-CAs have been described (Fett and Coleman, 1994). At this time sequences encoding at least five β-CA genes from A. thaliana are in the EST database but only the stroma and cytoplasmic locations of these proteins have been confirmed. All β-CAs have a histidine and two cysteine residues that act as zinc ligands (Hewett-Emmett and Tashian, 1996). β-CAs are generally more insensitive to inhibition by sulfonamide compounds than the α-CAs (Hewett-Emmett and Tashian, 1996). More information about β-CAs may be found in Chapter 1.

Two mitochondrial β-CAs have been identified previously in the green alga C. reinhardtii (Eriksson et al., 1996). Mitochondrial β-CAs are induced strongly under low CO₂ but
not under high CO₂ conditions (Eriksson et al., 1998). Here I report the identification a new β-CA gene, Cah6, in *C. reinhardtii*, and partial characterization of the recombinant Cah6 protein. The Cah6 protein is targeted to the chloroplast stroma. Cah6 is expressed both under high (5% CO₂) and low CO₂ (0.035% of CO₂ in air) conditions but is slightly upregulated under low CO₂ conditions.

**RESULTS**

1. **Screening of the Cosmid and cDNA Library for Genomic and cDNA Clones of Cah6**

   To find new β-CA genes, the mitochondrial β-CA protein (Ca1) sequence of *C. reinhardtii* was used to BLAST the EST database of *C. reinhardtii*. The search yielded many ESTs that were the gene products of the mitochondrial β-CA genes. However, several other ESTs also were found that, while encoding a β-CA, did not belong to either of the two known mitochondrial β-CA genes. These ESTs were from the same gene and were analyzed by CAP (contig assembly program; http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl) to form a consensus Cah6 sequence. In order to amplify Cah6, several PCR primers were designed based on this Cah6 contig. The alignment and sequences of all Cah6 primers used for PCR and sequencing of Cah6 are given in Appendix 1 of this dissertation.

   The PCR primers F4 and R5 (Appendix 1), were used on an indexed cosmid library to isolate a cosmid carrying the Cah6 gene. After several rounds of screening by PCR, two cosmid clones designated, 72-E-6 and 29-D-12, were isolated. Positive cosmid clones were verified as Cah6 clones after each round of screening by PCR, followed by sequencing of the PCR product using different Cah6 primers (Appendix 1). PCR using primers F4 and R5 yielded a product of 2.8 kb when used on the isolated cosmid clones (Fig. 4.1). The same two primers used on the cDNA core library yielded a PCR product of 2.4 kb which is six base pairs short from being a full length Cah6 cDNA clone [excluding the poly A tail; (Fig. 4.2)]. Detailed information on the
Figure 4.1 The screening of the cosmid library using the F4 and R5 primers. Lanes 1 and 5 show the PCR results using the cosmid library. Lanes 2 and 3 show the PCR results in the absence of DNA. Lane 4 shows a 1 kb DNA ladder (NEB). 72-E-6 and 29-D-12 are the two cosmids isolated containing the *CaH6* gene.
Figure 4.2 The screening of the cDNA core library for a Cah6 cDNA clone using PCR.
Lane 1 shows the PCR result using the F4 and R5 Cah6 primers on the cDNA core library. Lanes 2 and 3 show the PCR results using only a single primer F4 and R5, respectively on the cDNA library. Lane 4 shows PCR result using F4 and R5 primers on cosmid 72-E-6. Lanes 5 and 6 represent the PCR products in the absence of DNA. Lane 7 contains a 1 kb DNA ladder (NEB).
Figure 4.3  The genomic map of *Cah6*. The orange block arrows represent the four exons while the lines connecting the block arrows represent the three introns. The start and stop codons are labeled by black lines. The numbers within the parentheses denote the start and stop codon positions on the map in base pairs.
screening of the cosmid library and preparation of a cDNA library can be found in Chapter 2 of this dissertation.

2. Sequencing and Homology Search

The cosmid clones 72-E-6 and 29-D-12, and the 2.4 kb cDNA PCR product mentioned above, were sequenced in both directions using primers shown in Appendix 1. The sequencing results were confirmed by the EST and genomic database of *Chlamydomonas*. The *Cah6* gene has four exons and three introns and is 2885 bp long (Fig. 4.3). The size ranges of the exons are from 93 bp to 1652 bp while the introns range from 75 bp to 188 bp long. The genomic map and the full genomic sequence are given in Fig. 4.3 and Fig. 4.4, respectively.

The *Cah6* cDNA is 2452 bp long (Fig. 4.5). It encodes a putative protein of 264 amino acids. It contains a translation start site at nucleotide 309 and a stop site at nucleotide 1101 (Fig. 4.6). It has a very long 3′ UTR, more than 1300 base pairs long. A restriction map of the full length cDNA clone of *Cah6* is shown in Fig. 4.7. Predictions based on various protein prediction programs (SORT P, CHLOR P AND TARGET P) listed under ExPasy tools (http://ca.expasy.org/tools/#translate), indicate that the Cah6 protein is targeted to the chloroplast because it includes a putative chloroplastic transit peptide of 39 amino acids. A protein database search using the Cah6 protein sequence showed that it is similar to β-CAs from *Escherichia coli*, green algae and higher plants with an amino acid identity of 23% to 34%. A multiple sequence alignment of the Cah6 protein with that of β-CAs from other green algae and higher plants shows that it contains the characteristic one histidine and two cysteine residues as zinc coordination residues, seen in the enzymatically active β-CAs (Fig. 4.8).

3. Cloning of the *Cah6* in an Overexpression Vector

*Cah6* was cloned into the expression vector pMal to study the properties of Cah6 protein and to raise an antibody against it, if Cah6 were found to be enzymatically active. Two different
Figure 4.4 The genomic sequence of Cah6. The genomic sequence is 2885 bp long. The exon sequences are in uppercase and the intron sequences are in lowercase. The start and stop codons are shown in bold red.
Figure 4.5  The full length cDNA sequence of *Cah6*. The cDNA sequence is 2452 bp in length. The start and stop codons are shown in bold red. The polyadenylation signal is shown in bold blue.
Figure 4.6  The amino acid translation of the Cah6 cDNA clone. This protein shows an open reading frame of 264 amino acids. The start and stop codons are in bold red. The putative transit peptide sequence is shown in bold green.
Figure 4.7 The restriction map of the Cah6 cDNA. The restriction enzyme sites are indicated by black lines. The nucleotide numbers within the parentheses indicate the cleavage sites of the enzymes.
Cah6
-------------VSEAQSAISFQPSRSNRS---SLEKINSLTDRASSPEQVQLQNNLDGNM
Ca1
-------------ASAVNKGCCRCCRGRVACMGACMPMRHLHAHPNSPDQGFLYRELGNK
Coccomyxa
----------MSAKTDADLSPPLEARNR
Spinach
TLKEDMAYEAAAAALCKKLLSEKGELENEAAKVAQITSELAADGTPSASYFVQRKIEKFI

Cah6
RFLDG-----AVAHPHQDFSRVQAIAKQKPLAAILGCADSRLPAEVFDQGFDFDVFVCRVAG
Ca1
RFVNNKPHDSHTPLDNRVKAAGQKPFANCMCDADSRVPEIIQDFGFDFDFVTRVAG
Coccomyxa
KWADE-----CAAKDSTYFKVGAQPELYIGCADSRVPAQLFNMAPGEVVFVQRNVG
Spinach
KFKKE-----KYEKPNYEGSQQAPFAMFACSDRVCPSHLDGEQGEAPMRNIA

Cah6
NIATP-----EEIASLEYAVDLGKVEMLGLHTRCGAVKAALSGKAFPGFIDTLVDH
Ca1
NIVTN-----EITASLEFGTAVLGSKVLMGHLCSAGAVATMNGAANVPGGVISL
Coccomyxa
NLVSNKD-----LNCMSCEYTVDHKLKIHILVCGHYNCACKAGLVWHPKTAGVINLWIS
Spinach
NMVPFDSDKYAVGVAIEYAVHLKVENIVVGHSAAGGKMSFPDAGPTTDFIED

Cah6
LDVAISRVNSMSAKHAQAIKDGDVDMLDRVVENKYYQCQRSVIQEQLQGNN---LL
Ca1
ISPACKKA-----QAGGDVQ---AIAENVKVQMEQLKVSPLVGLVEEGK-LKI
Coccomyxa
DVREVRDKN-----AALKHGLSADDGWKMVELNVEAQVFNCASPQTVQWAARQGPLSV
Spinach
WKICLPAK----HKVLFAEHQNATFAEQCTHEKEAVNVLGNNFPTPVFRDGKVKTK-LAL

Cah6
AGAVYDLDGTGKHVSSTKGGSSAE--------------------------264
Ca1
VGGVYDLDATGKTEIA-----------------------------267
Coccomyxa
HIYVVTPTGTLVKEKLKPIGMDAGALLRADKLOCHFCFSESLA227
Spinach
QGGYYDFVNGSFELWGLEYGLSPQSV---------------------319

Figure 4.8  The alignment of the Cah6 protein sequence with those of other well characterized β-CAs. Ca1 represents the mitochondrial β-CA. Mitochondrial β-Cal and β-Ca2 (sequence not shown in the alignment) are almost identical in amino acid sequence and have only one amino acid difference in their sequences. Coccomyxa represent the cytosolic β-CA from the symbiotic alga Coccomyxa. The leader sequence of spinach which is 98 bp long is not shown here. Active site residues are shown in bold red. * represent a completely conserved amino acid, : represent conserved amino acid substitutions, and . represent semi conserved amino acid substitutions.
MalE-Cah6 recombinant overexpression constructs were generated. One contained the cDNA sequence coding for the full length open reading frame (ORF) of the Cah6 protein and the other contained the cDNA sequence that would code for the mature Cah6 protein predicted by the web protein prediction programs. The SP2 + R4H primers and the MP + R4H primers were used to amplify the cDNA coding for the full length and putative mature Cah6 proteins, respectively (Fig. 4.9).

Amplified cDNAs were purified from the gel and cloned into the overexpression vector pMal-c2x vector (Fig. 4.10) to generate two different types of recombinant constructs as described in Chapter 2. An in-frame insertion of Cah6 with the sequence of MBP in the recombinant clone was verified by restriction enzyme digestion analyses (Fig. 4.11) and DNA sequencing of the inserted Cah6 cDNA sequence. The latter was done to check for any missense or null mutation that could have been introduced by the DNA polymerase during PCR. Two clones were selected out of the twenty five recombinant clones of each type. Clone B48 had a cDNA insert coding for the full length Cah6 protein while clone B3 had one coding for the mature Cah6 protein.

4. Overexpression of the MBP-Cah6

*Escherichia coli* cells harboring the recombinant B48 and B3 recombinant constructs were induced with 1 mM IPTG for 2 hours at 37°C to overexpress the MBP-Cah6 fusion protein (Chapter 2). Equal amounts of proteins from induced and uninduced cells were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis (Fig. 4.12). The overexpressed recombinant Cah6 fusion protein was 15% of the total *E. coli* cell protein.

5. Purification and Activity Assays of the Recombinant Cah6

The crude cell extracts of B48 and B3 clones were used for CA activity assays. CA activity was detected in the cell extracts of the induced B48 clone but not in that of the B3 clone.
Figure 4.9  PCR for the construction of the pMal-Cah6 overexpression plasmid.  A. Lane 1 shows the PCR result using SP2 and R4H primers on the cDNA library.  Lane 2 shows a 1 kb DNA ladder.  Lane 3 shows the PCR result using the same set of primers in the absence of DNA.  B. Lane 1 shows a 1 kb DNA ladder (NEB).  Lanes 2, 3 and 4 (replicates) show the PCR results using the MP and R4H primers on the cDNA library.
Figure 4.10  A schematic diagram of the recombinant pMal-Cah6 expression construct.  A. A schematic figure showing the alignment of primers used to amplify the Cah6 cDNA. R4H primer has a HindIII site incorporated at the 5’end. The numbers on the top denote the primer position in base pairs. B. Part of the pMal vector showing the polylinker cloning site (PCS), β-galactosidase (LacZα) and β-lactamase (Ampγ) genes, XmnI and HindIII recognition sites. "A" denotes the Factor Xa cleavage site. MalE codes for the maltose binding protein (MBP). C. A schematic figure of the Cah6 recombinant construct.
Figure 4.11 The restriction enzyme digestions of constructs B48, B3 and pMal vector to verify the cloning of Cah6. A. Lane 1 contains a 2-log DNA ladder (NEB). Lane 2 contains uncut pMal. Lane 3 contains double digested pMal. Lane 4 contains uncut B48. Lane 5 contains double digested B48. B. Lanes 1, 2 and 3 contain double digested pMal, B48 and B3 plasmids, respectively. Lane 4 contains a 1 kb DNA ladder (NEB).
Fig 4.12  A 12% SDS-polyacrylamide gel showing the overexpression of the recombinant MBP-Cah6. Lane 1 represents the prestained molecular weight markers. Lanes 2 and 3 represent 30 µg of proteins from uninduced and induced *Escherichia coli* cells, respectively. The B48 clone was used for overexpression.
The B48 clone was selected for Cah6 purification. This clone had the entire ORF (open reading frame) of Cah6. The overexpressed recombinant Cah6 protein was purified by affinity chromatography using amylose resin according to a protocol given in the NEB technical catalogue. Purified recombinant Cah6 was further concentrated by using a 100 kDa cut-off centricon column.

At each step of purification the CA activity in the sample was assayed to check the purity of the Cah6 sample (Table 4.1). The recombinant Cah6 protein was found to have a specific activity of 400 WAU/mg. This calculation of specific activity was based on the total amount of recombinant fusion protein in the sample. CA activity assays were done using the method of Wilbur and Anderson (Wilbur and Anderson, 1948). CA activity was not detected in the extracts from uninduced cells containing the B48 clone or E. coli cells containing only the pMal vector.

The fusion protein purified from the B48 clone was cleaved by the protease Factor Xa for four hours at 23°C to separate Cah6 from the MBP. Factor Xa cleaved the fusion protein to yield 42 kDa MBP and 31 kDa Cah6 protein. It also nonspecifically cleaved the 31 kDa Cah6 protein band to yield a 28 kDa fragment. Use of common protease inhibitors like leupeptine did not prevent this nonspecific cleavage of Cah6. Purification and cleavage of the fusion protein was confirmed by performing SDS-PAGE (Fig. 4.13). There was no significant difference between the CA activities of the Factor Xa cleaved MBP-Cah6 fusion protein and uncut fusion protein. The 31 kDa Cah6 protein band was excised from the gel to be used as an antigen for production of polyclonal Cah6 primary antibodies.

6. Western Blotting and Northern Blotting Analyses of the Cah6 Expression

To test the specificity of the Cah6 antibody, Factor Xa (protease) cleaved MBP-Cah6 and purified MBP-Cah6 fusion proteins were separated by 12% SDS-PAGE and probed with the Cah6 antibody (Fig. 4.14). The antibody did not react with the MBP when induced E. coli cells
Table 4.1  The purification of the chimeric MBP-Cah6 from 2 liters of *E. coli* culture

<table>
<thead>
<tr>
<th>STEP</th>
<th>TOTAL ACTIVITY (W AU)</th>
<th>PROTEIN (mg)</th>
<th>SPECIFIC ACTIVITY (W AU/mg)</th>
<th>RECOVERY (%)</th>
<th>PURIFICATION FOLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated cells</td>
<td>1200</td>
<td>8000</td>
<td>0.15</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Amylose column</td>
<td>800</td>
<td>3</td>
<td>300</td>
<td>67</td>
<td>2000</td>
</tr>
<tr>
<td>Centricon column</td>
<td>400</td>
<td>1</td>
<td>400</td>
<td>33</td>
<td>2567</td>
</tr>
</tbody>
</table>

*a* one W AU = (t₀-t)/t where t₀ is the time for uncatalyzed reaction and t is the time for the enzyme catalyzed reaction; *b* determined by Lowry’s protein assay (Lowry *et al*., 1951)
Figure 4.13  A 12% SDS-polyacrylamide gel showing the uncut and the Factor Xa cut purified MBP-Cah6 protein. Lane 1 contains 55 µg of uncut purified recombinant protein. Lane 2 contains 55 µg of purified recombinant protein cut by 1 µg of Factor Xa. Lane 3 contains 1 µg of Factor Xa. Lane 4 contains the prestained molecular weight markers. Protein was purified from the B48 clone.
containing only the pMal-c2x vector or Factor Xa cleaved purified MBP-Cah6 fusion protein were used.

Proteins extracted from high CO\textsubscript{2} and air adapted D66 cells were separated by electrophoresis and probed with the Cah6 and mitochondrial $\beta$-CA primary antibodies (Fig. 4.15). The Cah6 antibody detected the Cah6 protein (28 kDa band) in both the high CO\textsubscript{2} and air adapted cells. The air acclimated cells showed slight upregulation of the Cah6 protein compared to that of the high CO\textsubscript{2} adapted cells (Fig. 4.15A). The mitochondrial $\beta$-CA antibody picked up the 22 kDa mitochondrial $\beta$-CA protein in the air acclimated cells but not in the high CO\textsubscript{2} grown cells (Fig. 4.15B) in agreement with earlier observations (Eriksson et al., 1998).

The primers X-9 and R5 (Appendix 1) were used to amplify an 826 bp PCR product from the cDNA core library (Fig. 4.16). This 826 bp PCR product is contained within the 3’ UTR of Cah6 and was used as a probe for Northern blot analyses using mRNA extracted from high and low CO\textsubscript{2} adapted Chlamydomonas (strain D66) cells grown in minimal medium (Fig. 4.16). Twenty µg of RNA from both cell types were loaded on the RNA gel. Northern blotting shows that Cah6 is expressed under both low and high CO\textsubscript{2} conditions, but is slightly upregulated under air levels of CO\textsubscript{2} cells.

7. Immunolocalization of Cah6

Air adapted D66 cells grown in minimal medium were used for immunolocalization of Cah6 following the protocol described in Chapter 2. Chlamydomonas cell sections were probed with the Cah6 antibody or the preimmune serum and observed under a transmission electron microscope (Fig. 4.17, Fig. 4.18 and Fig. 4.19). Immunogold densities in different cell compartments are given in Table 4.2. Immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. Immunolocalization results demonstrated that
Cah6 is located in the stroma of the chloroplast and is four fold more abundant in the area around the pyrenoid (particularly in the starch sheath of the pyrenoid; Fig. 4.18) compared to the other areas of the stroma.

8. Partial Characterization of the Cah6 Activity

The effects of sulfonamide and anion inhibitors on the CA activity of recombinant Cah6 were studied. Table 4.3 shows the inhibition of recombinant Cah6 by sulfonamides and anions. Generally, all β-CAs are less sensitive to sulfonamide inhibition and slightly more inhibited by anions like ClO₄⁻, I⁻, NO₂⁻, NO₃⁻, N₃⁻ etc than are α-CAs (Johansson and Forsman, 1993). Cah6 is no exception. Cah6 was comparatively less inhibited by the sulfonamides and more inhibited by the anions azide and cyanide than bovine CAII which is an α-CA. The effects of different temperatures on the CA activity of the recombinant Cah6 were also studied. The thermostability profile of Cah6 is shown in Fig. 4.20. The enzyme lost more than 50% of its activity above 43°C and completely lost its activity at 50°C. The optimum Cah6 activity was obtained at 33°C. Experiments also were performed with different thiol reducing agents to test the sensitivity of recombinant Cah6 to oxidation. β-CAs localized to higher plant chloroplasts have been reported to be sensitive to oxidation and therefore are dependent on a reducing environment to retain catalytic activity (Tobin, 1970; Atkins et al., 1972; Cybulsky et al., 1979; Johansson and Forsman, 1993). For example, oxidized pea CA requires a reducing agent for maximal activation of the enzyme (Johansson and Forsman, 1993). In contrast, the activity of Cah6 was found to be independent of a reducing agent. The effects of 10 mM 2-mercaptoethanol, cysteine and dithiothreitol on the Cah6 activity are given in Table 4.5.

9. Application of RNA Interference (RNAi) to Study the Functional Role of Cah6

I used RNAi to generate Cah6 mutants that would lack or underexpress the Cah6 protein. Such mutants would help to probe the functional role played by Cah6 in CCM and
photosynthesis. Two different RNAi constructs of Cah6 were generated by cloning reverse complementary sequences from the 5’ and the 3’ region of Cah6 in the pSL72 vector. The 5’ and the 3’ end regions of Cah6 that were least similar in DNA sequence to those of the mitochondrial β-CAs (Ca1 and Ca2) were selected based on comparative DNA sequence analyses of the Ca1, Ca2 and Cah6 genes. This was done to specifically underexpress Cah6 gene and not the other two mitochondrial β-CA genes, which have DNA sequences very similar to that of Cah6 in the coding regions of the gene. RNAi primers (Appendix 2) were designed to amplify reverse complementary fragments of Cah6 from the cDNA core library and cosmid 72-E-6. The alignment of these primers and the PCR results obtained using these primers are shown in Fig. 4.21 and Fig. 4.22, respectively. The 3’ end of the RNAi construct had reverse complementary sequences from a selected region in the 3’ UTR of the Cah6 cDNA (Fig. 4.23). The 5’ end RNAi construct contained a genomic sequence from the 5’ end of the Cah6 gene and the corresponding cDNA sequence cloned into the 5’ and 3’ end of the cytochrome c6 intron, respectively, in the pSL72 vector (Fig. 4.23). The genomic fragment had two introns of Cah6.

Six 5’ RNAi (5A, 7A, 8A, 10A, 11A and 13A) and five 3’ RNAi clones (1B, 7B, 8B, 10B and 13B) were selected. Double restriction enzyme digestions of these clones with AvrII and XbaI led to the identification of the positive 5’ and 3’ RNAi clones (Fig 4.24). Five 5’ (5A, 7A, 8A, 10A and 11A) and two 3’ RNAi positive clones (1B and 10B) were identified (Fig 4.24). 5’ RNAi clone 11A and 3’ RNAi clone 10B were selected as sources of RNAi plasmids for the transformation of wild type C. reinhardtii cells.

Transformation was done using the electroporation method described in Chapter 2. Both linear and circular forms of DNA from the RNAi clones were used for transformation. DNA was linearized by restriction digestion with Kpn1. Transformants were plated on TAP-paromomycin plates under diffuse light.
Three hundred 5´ RNAi and 600 hundred 3´ RNAi paromomycin resistant colonies were picked and regrown on fresh TAP-paromomycin plates. After one week, these colonies were screened on high and low CO₂ conditions on MIN plates. At this point there is no difference in the phenotype of the selected transformants under high and and low CO₂ conditions compared to that of the wild type C. reinhardtii cells. Screening of these transformants by Western blotting using the Cah6 antibody is being done currently. At this point, 5-6 air acclimated transformants of both types have been screened using Western blotting. None of them show any visible difference in the expression of Cah6 protein in the cells compared to that of in the wild type cells.

DISCUSSION

Here I report the identification of a nuclear gene encoding a novel chloroplastic β-CA, Cah6, and the partial characterization of the Cah6 protein in C. reinhardtii. β-CAs were the first CAs recognized in photosynthetic organisms (Burnell et al., 1990; Fawcett et al., 1990) but later have been identified in eubacteria, cyanobacteria, yeast, micro-algae and higher plants. They play important roles in these organisms. For example, β-CA has been reported to play an important role in the cyanobacterial CCM (Price and Badger, 1989b). In these organisms a carboxysomal β-CA, coded by icfA gene, converts HCO₃⁻ to CO₂ (Badger and Price, 1992). This increases the concentration of CO₂ at the site of Rubisco, ensuring efficient CO₂ fixation.

Alternatively, it has been suggested that the mitochondrial CAs (Ca1 and Ca2) of Chlamydomonas reinhardtii may play roles in recycling both respiratory and photorespiratory CO₂ by converting it to HCO₃⁻ in the mitochondrial matrix (Raven, 2001). This HCO₃⁻ then would leak back into the cytosol where it would be available for transport into the chloroplast stroma. This model assumes the absence of CA from the cytosol. Recently, it has been shown that the expression of mitochondrial CAs (Ca1 and Ca2) decreases when the external NH₄⁺ concentration decreases, to the point of being undetectable when the supply of NH₄⁺ restricts the
Figure 4.14  A Western blot probed by the Cah6 antibody using the purified overexpressed MBP-Cah6 protein. Lane 1 contains 20 µg of purified uncut fusion protein. Lane 2 contains 20 µg of fusion protein cleaved by 1 µg of Factor Xa. Lane 3 contains 1 µg of Factor Xa. ST represents prestained low molecular weight markers.
Figure 4.15 Western blots probed by the Cah6 and mitochondrial β-CA antibodies using the wild type *Chlamydomonas* cells. A. A Western blot probed by Cah6 antibody using high and low CO$_2$ adapted wild type *Chlamydomonas* (strain D66) grown in minimal medium cells. B. A Western blot probed by mitochondrial β-CA antibody using high (5%) and low CO$_2$ (0.03%) adapted wild type *Chlamydomonas* (strain D66) cells grown in minimal medium.
Fig 4.16  The Northern blot analyses of Cah6 expression. A. A schematic figure showing the alignment of primers (X-9 and R-5) used for making the probe for Northern blot analyses of Cah6 expression. B. A stained RNA gel showing RNA extracted from high (5%) and low CO\textsubscript{2} (0.03%) adapted wild type D66 cells grown in minimal medium. Each lane contains 20 µg of RNA. C. Northern blot result using the 826 bp PCR product as a probe.
Figure 4.17 A transmission electron micrograph showing the immunogold labeling of *C. reinhardtii* cells probed with the Cah6 antibody. Cells grown under low CO$_2$ (0.03%) conditions in minimal medium were probed with the Cah6 antibody. Ss, Py and C denote starch sheath, pyrenoid and chloroplast, respectively. Immunogold labelings are shown by black arrows.
Figure 4.18 A transmission electron micrograph showing the immunogold density around the pyrenoid in *C. reinhardtii* cells probed with the Cah6 antibody. Cells grown under low CO$_2$ (0.03%) conditions in minimal medium were probed with the Cah6 antibody. Ss, Py and C denote starch sheath, pyrenoid and chloroplast, respectively. Immunogold labelings are shown by black arrows.
Figure 4.19  A transmission electron micrograph showing the immunogold labeling of *C. reinhardtii* cells probed with the preimmune serum. Low CO$_2$ (0.03%) adapted cells grown in minimal medium were used. SS, P and C denote starch sheath, pyrenoid and chloroplast, respectively. Immunogold labelings are shown by small black arrows.
<table>
<thead>
<tr>
<th>Location</th>
<th>Area (µm²)</th>
<th>Immunogold density (# of immunogold particles / µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immune</td>
<td>Preimmune</td>
</tr>
<tr>
<td>Outside</td>
<td>7.80 ± 0.38</td>
<td>1.67 ± 0.10</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>6.84 ± 1.2</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.36 ± 0.05</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>Stroma</td>
<td>5.93 ± 1.07</td>
<td>3.0 ± 0.14</td>
</tr>
<tr>
<td>Pyrenoid</td>
<td>1.85 ± 0.30</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td>Starch sheath</td>
<td>0.86 ± 0.11</td>
<td>10.9 ± 1.9</td>
</tr>
</tbody>
</table>

Immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. The data presented in the table is the average ± SD of 15 cell sections. The stroma includes the thylakoid area. The cytoplasmic area was calculated by subtracting the total area of the chloroplast and nucleus from the cell area. The Cah6 antibody dilution used for immunolocalization was 1:10. The cells used for immunolocalization are air adapted D66 cells grown in minimal medium.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$I_{50}$ of bovine CA (M)</th>
<th>$I_{50}$ of Cah6 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>$1.4 \times 10^{-8}$</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>Ethoxyzolamide</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Azide</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Cyanide</td>
<td>$4.9 \times 10^{-5}$</td>
<td>$5 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

Erythrocyte bovine CAII was purchased from Sigma. The $I_{50}$ value corresponds to the concentration giving 50% inhibition. $I_{50}$ was determined by plotting percentage of inhibition vs. concentration of inhibitor. Sodium salts of azide and potassium salts of cyanide were used. The data shown for each treatment in the table are the averages ± SD of three different measurements.
Figure 4.20 **The thermostability of Cah6 activity.** Cah6 was incubated for 15 minutes at the indicated temperatures and cooled on ice. CA activity was measured at 4°C by the Wilbur-Anderson method. Activity is represented as a percentage of the activity of a sample maintained on ice throughout the experiment (400 WAU/mg). The percentage of Cah6 activity data for each temperature treatment shown in the graph are the averages ± SD of three sample evaluations.
Table 4.4 The effects of the SH-reducing agents on Cah6 activity

<table>
<thead>
<tr>
<th>SH-reducing agents</th>
<th>% of Cah6 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox-CA</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>100 ± 0.58</td>
</tr>
</tbody>
</table>

Cah6 was purified without any reducing agents in the buffer and the activity was measured. The specific activity (400U/mg) in the oxidized state is denoted as 100% activity (ox-CA). Prior to the measurements of CA activity, purified recombinant Cah6 enzyme was incubated for 30 minutes at room temperature with: 10 mm 2-mercaptoethanol, 10 mM Cysteine and 10 mM dithiothreitol. The data shown for each treatment in the table are the averages ± SD of three different measurements.
Figure 4.21 The genomic and cDNA maps of Cah6 showing the alignment of Cah6 RNAi primers. The orange block arrows and the lines interconnecting them represent the four exons and three introns, respectively. 5’ end and 3’ end RNAi primers are shown by black and red arrows, respectively. The numbers within the parentheses beside the primers denote the positions of the primers in base pairs.
Figure 4.22  PCR product generated using the Cah6 RNAi primers. The PCR primers are given at the bottom of each lane. Lane 1 is the PCR product using the cosmid 72-E-6 DNA. Lanes 2, 4, 6, 8 and 10 are the PCR results in the absence of DNA. Lanes 3, 5, 7, 9 represent PCR using the cDNA core library. X9 and R5 are the primers that were used to amplify an 826 bp cDNA product. This product was used as a probe for Cah6 Northern blot analyses. Lane 11 represents 1 kb ladder (NEB).
Figure 4.23 RNAi constructs of Cah6. A. 3’end RNAi construct. B. 5’end RNAi construct. The reverse complementary fragments of Cah6 were cloned in the pSL72 vector which is 5495 bp long. Restriction enzymes used to cut the vector and the Cah6 DNA fragments are shown by black lines. *AphVIII* and *Amp* codes for paromomycin and ampicillin resistance, respectively. *PsaD* is a constitutive promoter. The intron represented by the green box is the second intron of the cytochrome c6 gene of *C. reinhardtii*. Pink boxes in the 5’ end of Cah6 gene represent two introns.
Figure 4.24 The restriction enzyme digestions of *Cah6* RNAi clones. AvrII and XbaI were used for double restriction digestion. The RNAi clone number is denoted by a number followed by an alphabet. “A” and “B” represent 5´ and 3´ RNAi constructs respectively. ST represents the DNA 2-log ladder (NEB).
rate of photoautotrophic growth (Giordano et al., 2003). The expression of these CAs was induced at 0.2% CO$_2$ condition by increasing the NH$_4^+$ concentration in the growth medium. These workers have proposed that the mitochondrial CAs are involved in supplying HCO$_3^-$ for anaplerotic assimilation catalyzed by phosphoenolpyruvate carboxylase, which in turn provides carbon skeletons for nitrogen assimilation under certain conditions.

Other examples include the fact that deletion of the β-CA-like gene NCE103 of the yeast _S. cerevisiae_ causes an oxygen-sensitive growth defect (Götz et al., 1999). It has also been found that the tobacco salicylic acid-binding protein 3 (SABP3) is a chloroplast β-CA which exhibits antioxidant activity and plays a role in the hypersensitive defense response (Slaymaker et al., 2001). In _E. coli_, the cynT gene which codes for a β-CA is a part of the cyn operon; this β-CA recycles the CO$_2$ produced in the reaction of cyanate with HCO$_3^-$, back to HCO$_3^-$, which would have diffused out of the cell otherwise (Guilloton et al., 1993). Finally, a β-CA gene identified in _Corynebacterium glutamicum_ has been shown to be essential for achieving normal growth under atmospheric conditions (Mitsuhashi et al., 2003). These workers have shown that the effect of this β-CA is most likely due to its ability to maintain favorable intracellular HCO$_3^-$ levels, particularly during exponential growth phases and also in the case of L-lysine overproduction, both of which are conditions of higher HCO$_3^-$ demand (Mitsuhashi et al., 2003).

_Cah6_ is the third β-CA and the sixth CA gene to be identified in _C. reinhardtii_. The Cah6 protein has two cysteine and one histidine residues as zinc coordinating ligands, similar to all known enzymatically active β-CAs (Moroney et al., 2001). It is less susceptible to sulfonamide inhibitors than the α-CA from bovine erythrocytes, a characteristic trait of all active β-CAs. In general, β-CAs have sulfonamide I$_{50}$ ranging from 2 µM to 10 µM. The I$_{50}$ of Cah6 falls within this range. The full length protein has a calculated molecular weight of 28 kDa and a pI of 7.0 (predicted by various protein prediction programs listed under the ExPASY website).
has an apparent weight of 31 kDa on a SDS-polyacrylamide gel. It is similar to β-CAs from *E. coli*, green algae and higher plants with an amino acid identity of 23% to 34%, and most closely resembles the green alga *Coccomyxa* β-CA with a 34% identity. Like the activity of the *Coccomyxa* β-CA, that of Cah6 is unaffected by SH-reducing agents. The enzyme lost more than 50% of its activity above 43°C and became inactive at 50°C, with an optimal activity around 33°C. Thus the optimal temperature for Cah6 activity, like that of Cah3, falls within the range of the optimal growth temperature (12°C-35°C) of *C. reinhardtii*. The mature Cah6 protein (lacking the 39 amino acid residues that comprise the signal peptide of Cah6) has a calculated molecular weight of 24 kDa and a pI of 6.58. It has an apparent molecular weight of 28 kDa on a SDS-polyacrylamide gel.

CA activity was detected in cell extracts of the induced B48 clone but not in that of the B3 clone of *E. coli*, expressing the Cah6 gene. The B48 clone contains the entire ORF of Cah6 whereas the B3 clone had only the cDNA coding for the mature Cah6 protein. Eukaryotic proteins overexpressed in *E. coli* cells usually do not undergo post translational modifications, seen in a eukaryotic cell. The mature Cah6 protein (without the transit peptide) expressed in the B3 clone had a higher chance of getting properly folded than that (full length protein) expressed in the B48 clone. Under this circumstance protein from the B3 clone would be expected to be enzymatically more active than that from the B48 clone. If the overexpression of the Cah6 is toxic to *E. coli* cells, bacteria can either degrade the overexpressed protein or specifically introduce mutations in the DNA sequence coding for the toxic protein (personal communication, Dr. S.G. Bartlett, Louisiana State University, Baton Rouge, Louisiana). Thus the original B3 clone that I had selected by DNA sequencing (before measuring activity assays), could have got lost in the course of selection of the clones that had the mutated Cah6 protein. This can explain the lack of CA activity in the protein from B3 clone. In an effect to confirm this hypothesis,
resequencing of the *Cah6* gene in the B3 clone will be necessary in order to eliminate the possibility for any mutations introduced by the bacteria.

Alternatively, there can be another explanation for the lack of activity of the protein from B3 clone. The first amino acid in the mature Cah6 is an arginine. Cloning of the Cah6 cDNA coding for mature Cah6 in B3 clone was done by eliminating the codon for arginine as Factor Xa does not cleave any protein that starts with an arginine after the Factor Xa recognition sequence [Ile-(Glu-Asp)-Gly-Arg]. Thus mature Cah6 protein in the B3 clone started with a serine instead of an arginine residue. If no mutations are found in the *Cah6* sequence in B3 clone, then site directed mutagenesis experiments may be used to alter the arginine residue in the enzymatically active Cah6 from the B48 clone in order to study whether or not the single arginine residue in Cah6 does play a critical role in modulating the catalytic activity of Cah6.

Western and Northern blotting analyses show that Cah6 is constitutive and slightly upregulated under low CO\(_2\) conditions. Immunolocalization shows that Cah6 is localized in the stroma of the chloroplast and is not present in the pyrenoid. Interestingly, in *C. reinhardtii* immunogold density is four fold higher in the area around the pyrenoid, particularly around the starch sheath which surrounds the pyrenoid, compared to that in the other areas in the stroma of the chloroplast (Table 4.2). Based on these observations, I hypothesize that Cah6 indirectly plays a role in the CCM by trapping CO\(_2\) diffusing out from the pyrenoid, the site of localization of Rubisco in *C. reinhardtii*, and converting it to HCO\(_3^−\) (Fig. 4.25). This conversion of CO\(_2\) to HCO\(_3^−\) would increase the HCO\(_3^−\) pool in the stroma, thereby promoting the Rubisco activity inside the pyrenoid. Generation of Cah6 mutants might provide insight into the role of Cah6 in the chloroplast of *C. reinhardtii*. At this point, none of the six RNAi air acclimated transformants screened show any visible difference in the expression of Cah6 protein in the cells compared to that in the wild type cells. Hence no deduction can be made about the functional
Figure 4.25  A model showing the potential role of Cah6 and other known CAs in the operation of CCM in C. reinhardtii. The font sizes of CO$_2$ and HCO$_3^-$ indicate the relative concentrations of these Ci species. cyCA represents cytoplasmic carbonic anhydrase. Cah1 and Cah3 represent the periplasmic and thylakoid CAs, respectively. Putative HCO$_3^-$ transporters are denoted by small brown circles.
role played by Cah6 in the CCM and photosynthesis. Further screening of the remaining air adapted transformants by Western blotting will provide definite indication if the RNA interference is in operation in the wild type *C. reinhardtii* cells.
CHAPTER 5
IDENTIFICATION, CLONING AND OVEREXPRESSION OF A 
γ-CA LIKE GENE (Gclp 1)

INTRODUCTION

The first γ-CA discovered was from the archaebacterium Methanosarcina thermophila (Alber and Ferry, 1994). Since that time, although genes encoding putative γ-CA proteins have been found in eubacteria and plants (Newman et al., 1994), γ-CA from M. thermophila is the only enzymatically active γ-CA known to date. One of the carboxysomal shell proteins in Synechococcus PCC7942 (Price et al., 1993), CcmM, has been found to have a strong N-terminal sequence homology with the CA active site from γ-CAs. The C-terminal region of CcmM has three to four 87 amino acid repeats that are very similar to the small subunit of Rubisco protein from the cyanobacteria. Cells deleted in CcmM show a HCR phenotype and have empty carboxysomes. From these results it is clear that CcmM is required for correct carboxysome assembly and plays an essential role in the CCM in cyanobacteria. It is not clear, however whether CcmM has CA activity, or if its enzymatic activity is needed for correct assembly of carboxysomes. Three Arabidopsis ESTs in the databases have homologies with the γ-CA from M. thermophila, but it is not yet known if any of the Arabidopsis γ-CA proteins have CA activity or what their physiological roles might be. More information about γ-CAs may be found in Chapter 1.

The Chlamydomonas EST database has two contigs that align well with the γ-CA of M. thermophila (Cam) and the cyanobacterial γ-CA homologue CcmM. These two genes encoding γ-CA like proteins have been termed Gclp1 and Gclp2, respectively. Gclp1 and Gclp2 have more than 50 and 40 ESTs, respectively, implying that they are highly expressed. In this chapter
I report my research on the *Gclp1* gene. This is the first report of a putative γ-CA gene in *C. reinhardtii*.

RESULTS

1. Screening of Cosmid and cDNA Libraries for Genomic and cDNA Clones of *Gclp1*

The γ-CA protein sequence of *M. thermophila* was used to BLAST the EST database of *C. reinhardtii*. The search yielded several ESTs that were analyzed by CAP (contig assembly program; http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl) to form the consensus *Gclp1* and *Gclp2* sequences. *Gclp1* was selected for further studies. In order to amplify *Gclp1*, several PCR primers were designed based on the *Gclp1* contig. The PCR primers, 1F and 2B, were used with genomic DNA to generate a product of 745 bp. This PCR product was used to make a probe to screen an indexed cosmid library. After three sequential rounds of screening, two cosmid clones, named 70-C-3 and 70-G-8, were isolated. Positive cosmid clones were verified as *Gclp1* clones, after each round of screening, by PCR followed by sequencing of the PCR product using different *Gclp1* primers. Primers M1F and M1R were used on the two cosmid clones to yield a PCR product of 2249 bp (Fig. 5.1). The same two primers used on a cDNA core library yielded a PCR product of 1170 bp (Fig. 5.1).

Detailed information on the screening of the cosmid library and preparation of cDNA library may be found in Chapter 2 of this dissertation. Sequences and the alignment of all *Gclp1* primers are given in Appendix 3 of this dissertation.

2. Sequencing and Homology Search

The cosmid clones 70-C-3 and 70-G-8, and the 1170 bp cDNA PCR product mentioned above, were sequenced in both directions using M1F and M1R primers. The sequencing results matched those in the *Chlamydomonas* EST and genomic databases. The *Gclp1* gene has seven exons and six introns and is 2374 bp long (Fig. 5.2). The size ranges of exons and introns are
from 69 bp to 583 bp and 103 bp to 429 bp, respectively. The full genomic sequence is given in Fig. 5.3. The Gclp1 cDNA is 1294 bp long (Fig. 5.4). A restriction map of the full length cDNA clone of Gclp1 is shown in Fig. 5.5. Gclp1 cDNA encodes a protein of 312 amino acids. It contains a translation start site at nucleotide 104 and a stop site at nucleotide 1039 (Fig. 5.6). Predictions based on various protein prediction programs (SORT P, CHLOR P AND TARGET P) listed under ExPasy server (http://ca.expasy.org/tools/#translate), indicate that it is either located in the cytoplasm or is secreted outside the cell. In the latter case, a putative transit peptide of 41 amino acids has been predicted. Gclp1 has 30%-40% homologies with γ-CA of M. thermophila and CcmM of cyanobacteria, and contains the characteristic three histidines which may serve as zinc coordination residues seen in the enzymatically active γ-CA of M. thermophila (Fig. 5.7).

3. Cloning of Gclp1 in an Overexpression Vector

Gclp1 was cloned in the overexpression vector pMal-c2x to study Gclp1 properties and to raise an antibody, if the Gclp1 was found enzymatically active. Two different pMal-Gclp1 recombinant overexpression vectors were constructed. One contained the cDNA sequence coding for the full length open reading frame (ORF) of the Gclp1 protein and the other contained the cDNA sequence that would code for the mature Gclp1 protein, if the first 41 amino acids were a leader sequence as predicted by the various protein prediction programs.

S1 + M1RH primers and S3 + MIRH primers were used to amplify the cDNA coding for the ORF and putative mature Gclp1 protein, respectively (Fig. 5.8). Amplified cDNAs were purified from the gel and cloned into the pMal vector (Fig. 5.9) to generate two different types of recombinant MalE-Gclp1 constructs as described in Chapter 2. The in-frame insertion of Gclp1 with the sequence of MBP in the recombinant clone was verified by restriction enzyme digestion analyses (Fig. 5.10) followed by DNA sequencing of the Gclp1 cDNA sequence cloned.
Figure 5.1 The screening of the cDNA and cosmid libraries for a cDNA and genomic \textit{Gclp1} clone using PCR. Lane 1 shows the PCR result using M1F and M1R $\gamma$-CA primers on the cDNA library. Lane 2 and Lane 3 show the PCR results using MIF and MIR primers on the genomic cosmids 70-C-3 and 70-G-8, respectively. Lane 4 and 6 represent PCR products in the absence of DNA. Lane 5 contains a 1kb DNA ladder.
**Figure 5.2 The genomic map of *Gclp1*.** The orange block arrows and lines connecting the block arrows represent the seven exons and six introns, respectively. The start and stop codons are labeled by black lines. The numbers within the parenthesis denote the nucleotide positions of the start and stop codons. *Gclp1* is 2374 base pairs long.
Figure 5.3  The genomic sequence of Gcpl1. The Gcpl1 gene is 2374 bp long. The exon sequences are in upper case and the intron sequences are in lower case. The start and stop codons are shown in bold red.
Fig 5.4  The full length cDNA sequence of Gclp1. The length of the cDNA is 1294 base pairs (excluding the Poly A tail). The start and stop codons are shown in bold red. The putative polyadenylation signals are in bold blue.
**Figure 5.5 The restriction map of the Gclp1 cDNA.** The restriction enzyme sites are indicated by black lines. The nucleotide numbers within the parentheses indicate positions of the cleavage sites of enzymes in base pairs.
Figure 5.6 The amino acid translation of the cDNA clone (Y-21) of Gclp1 encoding the full length protein. This protein has an open reading frame of 312 amino acids. The start and stop codons are in bold red. The putative transit peptide is shown in bold green.
Figure 5.7 The alignment of Gclp1 with other γ-CAs. The zinc coordination residues are shown in red. The γ-CA from *Methanosarcina thermophila* is Cam and the γ-CA homologue of *Synechococcus sp.* is CcmM. Cam has an open reading frame of 247 amino acids. The CcmM protein from *Synechococcus* has a long C-terminal extension. CcmM has an open reading frame of 539 amino acids. The Gclp1 protein from *Chlamydomonas reinhardtii* has an open reading frame of 312 amino acids. * represents a completely conserved amino acid, : represent conserved amino acid substitutions, and . represent semi conserved amino acid substitutions.
Figure 5.8  PCR for the construction of the *Gclp1* overexpression plasmid. A. Lane 1 and Lane 2 show PCR results using the S1 and M1RH primers on the cDNA library. Lane 3 shows PCR result using the same set of primers in the absence of DNA. Lane 4 shows a 1 kb DNA ladder. B. Lanes 1, 2, 4, 5 and 6 show PCR results using S3 and M1RH primers on the cDNA library. Lane 3 shows a 1 kb DNA ladder.
Figure 5.9  A schematic diagram of the recombinant pMal-Gclp1 expression construct. A. A schematic figure showing the alignment of primers (S1/S3 and M1RH) used to amplify Gclp1 cDNA. The M1RH primer has a HindIII site incorporated at the 5’ end. The numbers on the top denote the primer positions in base pairs. B. Part of the pMal vector showing the poly linker cloning site (PCS), β-galactosidase (LacZα) and β-lactamase (Amp′) genes, XmnI and HindIII recognition sites and Factor Xa cleavage site. MalE codes for the MBP (maltose binding protein). C. A schematic figure of the Gclp1 recombinant construct.
into the pMal-c2x vector. The latter was done to check for any missense or null mutation that could have been introduced by the DNA polymerase during PCR. Two clones were selected out of the thirty recombinant clones of each type. Clone Y-21 had the cDNA insert coding for the full length Gclp1 protein while clone Y-9 had the cDNA insert coding for the putative mature Gclp1 protein.

4. Overexpression and Purification of the Recombinant MBP-Gclp1 Fusion Protein

Both the clones were tested for overexpression of Gclp1. Recombinant E. coli cells were induced with 1 mM IPTG for two hours at 37°C for overexpression of the fusion protein. Equal concentrations of protein from induced and uninduced E. coli cells were loaded on a 12% SDS-polyacrylamide gel (Fig. 5.11). The overexpression of the recombinant full length Gclp1 was 30%-40% lower than that of Cah3 and Cah6 under identical inducing conditions. The overexpressed recombinant Gclp1 proteins were purified by affinity chromatography using amylose resin according to a protocol given in the NEB technical bulletin. The fusion proteins were cleaved by the serine protease, Factor Xa, for four hours at 23°C to separate Gclp1 from the MBP. Purification and cleavage of fusion protein was confirmed by performing SDS-PAGE (Fig. 5.12). Moreover, the Gclp1 protein was very susceptible to proteolytic degradation during purification. SDS-PAGE results showed that Factor Xa cleaved fusion protein (from Y-21 clone) specifically to yield MBP (42 kDa) and Gclp1 (36 kDa). Along with this specific cleavage, there was evidence of nonspecific cleavage of Gclp1 fusion protein yielding a fragment of 50 kDa and a fragment of 28 kDa. This nonspecific cleavage was enhanced in the presence of Factor Xa and was not prevented by the presence of the common protease inhibitors.

5. CA Activity Assays and Immunoblotting Results

No CA activity was detected in the crude cell extracts or Factor Xa cut and uncut purified fusion protein from either type of recombinant Gclp1 clone. Under the same CA activity assay
Figure 5.10  The restriction enzyme digestions of constructs Y-21, Y-9 and the pMal vector to verify cloning of $Gclp1$. Lanes 1 to 4 represent restriction digestions of Y-21 and pMal plasmids with SacI and HindIII. Lane 5 contains a 2-log DNA ladder (NEB). Lanes A to I represent restriction digestions of Y-9 and the pMal plasmids with SacI and HindIII. Lane J contains a 1 kb DNA ladder (NEB). Double digests of Y-21 and Y-9 with SacI and HindIII yield the DNA inserts of size 1.1 kb and 1 kb.
Figure 5.11  A 12% SDS-polyacrylamide gel showing the uninduced and induced *Escherichia coli* cells expressing the recombinant MBP-Gclp1 protein. Lane 1 and Lane 2 contain 30 µg of proteins from induced and uninduced *E. coli* cells, respectively. Lane 3 contains prestained molecular weight markers.
Figure 5.12  A 12% SDS-polyacrylamide gel showing the purified uncut and Factor Xa cut MBP-Gclp1 protein. Lane ST represents the prestained molecular weight markers. Lane 1 contains 20 µg of uncut chimeric MBP-Gclp1. Lane 2 contains 30 µg of chimeric MBP-Gclp1 cut by 1 µg of Factor Xa. Lane 3 contains 1 µg of Factor Xa. Proteins were stained with Coomassie Blue after 12% SDS-PAGE.
conditions, a crude extract of *E. coli* cells overexpressing Cam (γ-CA of *M. thermophila*) and the purified recombinant Cam showed significant amounts of CA activities with specific activities of about 0.30 WAU/mg and 800 WAU/mg, respectively. Crude recombinant *E. coli* cells overexpressing Gclp1 and Factor Xa cut and uncut purified recombinant Gclp1 fusion proteins were probed by the primary antibody raised against the γ-CA protein of *M. thermophila* in Western blotting. The immunoblotting showed that the antibody raised against the archaebacterial γ-CA protein did not cross react with Gclp1 (data not shown).

**DISCUSSION**

Here I report the identification of a putative γ-CA gene from *C. reinhardtii* for the first time. Since its discovery in the archaebacterium *Methanosarcina thermophila*, several putative γ-CA genes have been identified in eubacteria and plants. In the methanogen *M. thermophila*, a metabolic switch from methanol to acetate utilization results in the elevation of CA activity suggesting this enzyme is important for growth on acetate. It has been proposed that the CA might be required for a CH$_3$CO$_2$/H$^+$ symport system or for efficient removal of cytoplasmically produced CO$_2$ (Alber and Ferry, 1994). A γ-CA homologue, CcmM, was discovered earlier in *Synechococcus* PCC7942 (Price *et al.*, 1993). CcmM does not have any detectable CA activity (Moroney *et al.*, 2001) but is clearly required for optimal growth on low CO$_2$ (Price *et al.*, 1993).

The homology search analysis using protein prediction programs (P SORT, CHLOR P and TARGET P listed under [http://ca.expasy.org/tools/#translate](http://ca.expasy.org/tools/#translate)) show that Gclp1 is either located in the cytoplasm or is secreted outside the plasma membrane. It has three histidine residues that could serve as zinc coordination residues in the amino acid sequence, as seen in Cam. Gclp1 aligns well with CcmM and the *M. thermophila* γ-CA, Cam. The full length and mature Gclp1 have a calculated molecular weight of 28 kDa and 34 kDa, respectively. The calculated pI of the full length and mature Gclp1 are 6.54 and 6.36, respectively.
No detectable CA activity like that found in the uncut or Factor Xa cut purified recombinant Cah3 and Cah6 fusion proteins has been found in the heterologously produced recombinant Gclp1 fusion protein. Under the same CA activity assay conditions, crude extracts of *E. coli* cells overexpressing the γ-CA of *M. thermophila* (Cam) and purified Cam showed significant amount of CA activity. Although this seems to indicate that Gclp1 might be an enzymatically inactive CA in *C. reinhardtii*, there can be other explanations for the inactivity of the enzyme. For example, CcmM, the γ-CA homologue from *Synechococcus*, lacks detectable CA activity (Moroney *et al.*, 2001) but it is required for growth under low CO₂ conditions.

Comparisons of SDS-PAGE results of overexpression and purification of recombinant Cah3, Cah6 and Gclp1 fusion proteins indicate that the overexpression of recombinant Gclp1 was 30%-40% lower than that of Cah3 and Cah6 under identical inducing conditions. Moreover, the Gclp1 protein was susceptible to proteolytic degradation during purification (Fig. 5.12). This nonspecific cleavage was enhanced in the presence of factor Xa and was not prevented by the presence of common protease inhibitors. The above results suggest the possibilities that the Gclp1 protein is degraded as a response to possible toxicity to the *E. coli* cells, or that the chimeric protein is unstable.

On the other hand, it is also possible that the Gclp1 has become enzymatically inactive in the course of evolution. Gclp1 has 40%-43% amino acid identity with an APF1 transcription factor, a putative ferripyochelin binding protein in *Arabidopsis* and also to some proteins of unknown function in *Oryza sativa* and *Zea mays*. Hence, other functional roles of Gclp1 *in vivo* cannot be ruled out.

The *Chlamydomonas* EST database has another putative γ-CA homologue, Gclp2 which is 80% similar to Gclp1. The *Arabidopsis* genome contains at least three homologues of γ-CA; but it is not yet known if these gene products are active CAs or what physiological role they
might be playing in the cell. *Gclp1* and *Gclp2* have more than 40 ESTs. Northern blot analyses of these two genes using air and high CO$_2$ adapted cells can be used in future research to indicate if the expression of these genes is regulated by the CO$_2$ concentration in the air. Also, generation of mutants of these putative γ-CA genes by antisense RNA or RNAi might allow a better understanding of the functional roles of these genes.
CHAPTER 6
CONCLUSIONS

There has been a surge of interest in CAs from plants and algae over the past decade. This interest began with the discovery of the $\beta$-CA in plants in 1990 (Fawcett et al., 1990) and has continued with the finding of multiple $\alpha$- and $\beta$-CAs in Chlamydomonas reinhardtii and Arabidopsis thaliana and the determination of the critical physiological roles, CAs have in cyanobacteria and macro-algae. Analysis of the Arabidopsis genomic database reveals at least 14 genes, potentially encoding CAs, that have homologies with ESTs and are expressed in cells (Moroney et al., 2001). Clearly the number of CAs in plants is much greater than previously thought. C. reinhardtii, a unicellular green alga, is not far behind with five CAs already identified (Table 1.2; Moroney et al., 2001).

The primary goals of my work were to a) identify, clone and overexpress novel CA and CA-like genes from the green unicellular alga Chlamydomonas reinhardtii, b) partially characterize recombinant CA proteins if they are active, and c) determine if the new CAs play any role in photosynthesis and CCM of the green alga. In addition to characterizing the recombinant Cah3 protein encoded by the Cah3 gene, identified by Karlsson et al., (1995), I have identified one novel $\beta$-CA gene (Cah6), one new putative $\beta$-CA gene (Cah7) and two putative $\gamma$-CA like genes (Gclp1 and Gclp2) in C. reinhardtii (Table 6.1). This brings the total number of CA/CA-like proteins in C. reinhardtii to nine (Table 6.1). I have concentrated on Cah6 and Gclp1 for the research described herein.

Karlsson et al. (1998) published the sequence of the Cah3 cDNA. Although the Cah3 gene has been well characterized, there is no report of the activity of the purified protein. This is
<table>
<thead>
<tr>
<th>Gene family</th>
<th>Known gene/s</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Cah1, Cah2</td>
<td>periplasm</td>
<td>Fukuzawa et al. 1990; Fujiwara et al. 1990</td>
</tr>
<tr>
<td>α</td>
<td>Cah3</td>
<td>thylakoid</td>
<td>Karlsson et al. 1995</td>
</tr>
<tr>
<td>β</td>
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<td>mitochondria</td>
<td>Eriksson et al. 1996</td>
</tr>
<tr>
<td>β</td>
<td>Cah6</td>
<td>chloroplast**</td>
<td>This dissertation</td>
</tr>
<tr>
<td>β</td>
<td>Cah7</td>
<td>unknown</td>
<td>Gene model predictions and EST database</td>
</tr>
<tr>
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<td>Gclp1</td>
<td>cytoplasm*</td>
<td>This dissertation</td>
</tr>
<tr>
<td>γ</td>
<td>Gclp2</td>
<td>cytoplasm*</td>
<td>Gene model predictions and EST database</td>
</tr>
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</table>

* based on analyses by protein prediction programs PSORT, TARGET P and CHLOR P; ** based on immunolocalization results. The names of CA genes identified after 1998 are given in bold.
the first report of detection of significant CA activity in the purified Cah3 protein and partial characterization of this CA activity. α-CAs are known to have a broad range of specific activity ranging from 5 WAU/mg (human CAIII) to 2000 WAU/mg (human CAII). The specific activity of the recombinant Cah3 enzyme, which is around 735 WAU/mg, falls within this range.

Recombinant Cah3 was purified to raise a "new" Cah3 antibody that would be a more dependable tool for Western blotting and immunolocalization experiments than the existing “old” Cah3 antibody. Western blotting results using the new Cah3 antibody have shown that the protein is missing in the two allelic cah3 mutants, ca-1 and cia3 (Fig. 3.6, Chapter 3). The immunoblotting results also indicate that the “new” Cah3 antibody specifically detects the Cah3 and is more dependable as a tool in performing immunolocalization studies. The Cah3 enzyme requires maintenance in the oxidized state to retain its CA activity, as indicated by a reduction of activity following exposure to thiol reducing agents. This seems to indicate that disulfide bond formation in Cah3 is essential.

Researchers in several laboratories have tried to assay CA activity in chloroplasts. Using mass-spectrometric measurements of $^{18}$O exchange, Sültemeyer et al., (1995) have characterized two chloroplastic CA activities in C. reinhardtii cells. One CA activity is an “insoluble” form associated with the thylakoid fraction and is more insensitive to EZ while the other is a “soluble” form and sensitive to EZ (Amoroso et al., 1996). Villarejo et al., (2001) have found a new chloroplast envelope CA activity that is sensitive to EZ and is constitutively expressed. Support for the presence of the insoluble chloroplastic CA activity is provided by the identification of the 29 kDa Cah3 which is located on the luminal side of thylakoid membrane (Karlsson et al., 1998). I have found Cah3 to be sensitive to EZ, contrary to the finding of Sültemeyer et al., (1995).

My research data show that Cah6, the sixth carbonic anhydrase gene and the third β-CA gene to be identified in C. reinhardtii, is located in the stroma of the chloroplast and can serve as
the soluble chloroplastic CA but it is less sensitive to EZ contrary to the findings of Sültémeyer et al., (1995). Cah6 has about 20 ESTs in the data base at this time. Cah6 is less susceptible to sulfonamide inhibitors, a characteristic trait of all active β-CAs. β-CAs in higher plants are known to have specific activities ranging from 600 WAU/mg to 1000 WAU/mg. The specific activity of the recombinant Cah6 protein is 940 WAU/mg and falls within the activity range of β-CAs.

Both Cah3 and Cah6 are constitutive in expression but are slightly upregulated under low CO₂ conditions (Karlsson et al., 1998; Chapter 4 of this dissertation, 2003). Another mutant of C. reinhardtii, cia5, has a HCR phenotype, fails to induce CCM and does not synthesize any of the low CO₂ inducible mitochondrial and periplasmic CAs along with other low CO₂ inducible polypeptides. It is deficient in a putative transcription factor coded by the Cia5 gene (Fukuzawa et al., 2001; Xiang, et al, 2001). Katzman et al., (1994) used a ¹⁴C assay to measure the CA activity in the chloroplasts of cia5 and wild type cells of Chlamydomonas and found that the CA activity of cia5 is almost identical to that in wild type cells. The “new” Cah3 and Cah6 antibody detects the Cah3 and Cah6 protein, respectively in the cia5 cells (data not shown). So my research data on Cah3 and Cah6 agrees with the earlier work of Katzman et al., (1994). This also indicates that probably Cah3 and Cah6 gene expressions are not under the regulation of the Cia5 gene.

Comparative analyses of characteristic properties of Cah3 and Cah6 CA activities are shown in (Table 6.2). The sulfonamide I₅₀ of Cah6 was 100- to 1000- fold higher than that of Cah3, a characteristic trait of all β-CAs. The optimum temperatures for CA activity of Cah6 and Cah3 were around 32°C-33°C and 50% of the catalytic activities of these proteins were lost above 43°C (Table 6.2). Thus the optimal temperature for Cah6 activity, like that of Cah3, falls within the range of the optimal growth temperature (12°C- 35°C) of C. reinhardtii. β-CAs
localized to higher plant chloroplasts have been reported to be sensitive to oxidation and therefore are dependent on a reducing environment to retain their catalytic activity (Tobin, 1970; Atkins et al., 1972; Cybulsky et al., 1979; Johansson and Forsman, 1993). In contrast, the activity of Cah6 like that of the cytosolic $\beta$-CA of the green alga *Coccomyxa* was not affected by thiol reducing agents (Table 6.2). Cah6 also has 34% sequence identity with the cytoplasmic $\beta$-CA of *Coccomyxa*.

Immunolocalization results show that Cah6 is localized in the stroma of the chloroplast and is not present in the pyrenoid. Interestingly, it is four fold more abundant in the area around the pyrenoid, particularly in the starch sheath surrounding the pyrenoid than in the other areas in the stroma of the chloroplast. My hypothesis is that Cah6 might indirectly play a role in CCM by trapping any CO$_2$ leaking out from the pyrenoid (the site of localization of Rubisco in *C. reinhardtii*) into HCO$_3^-$ . This would help to maintain the HCO$_3^-$ pool in the stroma, which is essential for the operation of CCM.

RNA interference was used to generate Cah6 mutants to provide insight into the functional role of Cah6 in the chloroplast of *C. reinhardtii*. None of the RNAi transformants screened so far show any visible difference in the expression of Cah6 protein in the mutant cells compared to that in the wild type cells. Hence no deduction can be made about the functional role played by Cah6 in the CCM and photosynthesis based on this approach. Further screening of the air adapted cells of remaining transformants by Western blotting will provide definite indication as to whether or not RNA interference is working in the wild type *C. reinhardtii* cells. It might be possible even if the Western blotting results show that RNAi is working in these transformants, Cah6 mutants might not show any phenotypic difference from the wild type cells if it is playing an indirect role in CCM. Specific reduction of chloroplast CA activity by antisense RNA in transgenic tobacco plants had a minor effect on photosynthetic CO$_2$
assimilation (Price et al., 1994). Plants with even the lowest CA levels (2% of wild type levels) were not morphologically distinct from the wild type plants (Price et al., 1994). CA has a high turnover number and is one of the most catalytically efficient enzymes known to date. Hence it might be necessary to underexpress a CA gene to a level of 99%-100% in any RNAi CA mutant to see a visible phenotypic difference between the wild type and mutant cells.

Since its discovery in the archaebacterium Methanosarcina thermophila (Alber and Ferry, 1994), several putative \( \gamma \)-CA genes have been identified in eubacteria and plants. I have identified a putative \( \gamma \)-CA gene (Gclp1) in C. reinhardtii for the first time. The homology search and the multiple sequence analysis show that Gclp1 has significant homology to the one known active \( \gamma \)-CA (Cam) from M. thermophila and the putative \( \gamma \)-CA, CcmM from Synechococcus. It has three histidine residues that can function as zinc coordination residues in the amino acid sequence, a characteristic of \( \gamma \)-CAs. Gclp1 was cloned in the overexpression vector pMal-c2x. Like Cah6, two different expression constructs were made. One clone contained the ORF of Gclp1 and the other contained the cDNA sequence that would code for the mature Gclp1 protein. No detectable CA activity was found in the heterologously produced recombinant Gclp1 fusion protein from either clone. Under the same CA activity assay conditions, crude extracts of E. coli cells overexpressing Cam CA activity CA showed significant amount of CA activity. If the overexpression of the Gclp1 is toxic to E. coli cells, bacteria can die or can degrade the overexpressed protein or specifically introduce mutations in the DNA sequence coding for the toxic protein (personal communication, Dr. S.G. Bartlett, Louisiana State University, Baton Rouge, Louisiana). If expression of Gclp1 was toxic to bacteria, clones containing Gclp1 were lost and never selected. Alternatively, it can also be possible that the cells were overexpressing mutated Gclp1 or degrading the Gclp1 that they were expressing. Gclp1 was also highly susceptible to degradation. Any one of these could explain the lack of detectable activity.
There are quite a few questions that still remain to be answered to shed light on the intracellular locations and functional roles of these new CAs and the biochemical characteristics of CA activities. Analyses of Cah3 using various post translational prediction programs (PSORT, CHLOR P and TARGET P) suggest that the protein is located in the thylakoid. Thermolysin (a protease)-treated intact and sonicated thylakoids from wild type and cah3 mutant cia3 were analyzed by Western blotting using the “old” cah3 antibody (Karlsson et al., 1998). Previously published results using the “old” antibody showed that Cah3 is present on the luminal side of thylakoids and the mutant cia3 has the protein (Karlsson et al., 1998). Due to the lack of dependability of the “old” antibody, this experiment should be repeated with the “new” antibody. We are currently performing immunolocalization of Cah3 in WT137, cia3 and ca-1 cells, using this “new” antibody. The immunolocalization results along with the Western blotting analyses of thermolysin-treated intact and sonicated thylakoids from WT 137, ca-1 and cia3 will be helpful in reaching a definite conclusion regarding the intracellular location of Cah3 and its presence or absence in the two cah3 allelic mutants.

Cah3 has three cysteine residues in the mature protein. In the future, site directed mutagenesis experiments can be done to specifically substitute these cysteines in Cah3 in order to determine if these cysteines play a role in controlling the CA activity of Cah3, as they do in other α-CAs.

Furthermore, Cah3 (generated by cleavage of MBP-Cah3 fusion protein by Factor Xa and separated from MBP by SDS-PAGE) can be eluted from the polyacrylamide gel sections and the free thiol content of oxidized and reduced Cah3 enzyme estimated from the increase in absorbance (A_{412}) caused by formation of a 2-nitro-5 thiobenzoate anion resulting from cleavage of DTNB [5′, 5′-dithiobis (2-nitrobenzoic azide)] upon reaction with a thiolate anion (Hiltonen et al., 1998).
Table 6.2  The comparison of some characteristic properties of the recombinant Cah3 and Cah6 activity

<table>
<thead>
<tr>
<th>Biochemical traits</th>
<th>Mature Cah3</th>
<th>Cah6 (ORF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>730 WAU/mg</td>
<td>940 WAU/mg</td>
</tr>
<tr>
<td>Apparent molecular weight</td>
<td>29 kDa</td>
<td>31 kDa</td>
</tr>
<tr>
<td>pI</td>
<td>7.87</td>
<td>7.0</td>
</tr>
<tr>
<td>Thermostability</td>
<td>Loses 50% of CA activity at 43°C ± 0.7°C</td>
<td>Loses 50% of CA activity at 43°C ± 0.8°C</td>
</tr>
<tr>
<td>Effect of SH-reducing agent</td>
<td>Inhibits CA activity by 50% ± 1.4% to 82% ± 1.4%</td>
<td>No effect on CA activity</td>
</tr>
<tr>
<td>Sulfonamide Inhibition</td>
<td>EZ $I_{50}$ $(6 \times 10^{-9} ± 0.7 \times 10^{-9}$ to $8 \times 10^{-9} ± 0.9 \times 10^{-9}$M)</td>
<td>EZ $I_{50}$ $(2 \times 10^{-6} ± 0.3 \times 10^{-6}$ to $9 \times 10^{-6} ± 0.5 \times 10^{-6}$ M)</td>
</tr>
<tr>
<td>Azide Inhibition</td>
<td>$I_{50}$ $(3.2 \times 10^{-5} ± 0.2 \times 10^{-5}$ M)</td>
<td>$I_{50}$ $(1.5 \times 10^{-5} ± 0.4 \times 10^{-5}$ M)</td>
</tr>
<tr>
<td>Cyanide Inhibition</td>
<td>$I_{50}$ $(5.9 \times 10^{-5} ± 0.6 \times 10^{-5}$ M)</td>
<td>$I_{50}$ $(5 \times 10^{-6} ± 0.5 \times 10^{-6}$ M)</td>
</tr>
</tbody>
</table>

The data shown in the table for thermostability, effect of SH-reducing agents, sulfonamide, azide and cyanide inhibition are the averages ± SD of three different measurements. The specific activity calculated is calculated taking into account the molecular size ratios of Cah3 and Cah6 and MBP.
Some reasons for the CA encoded by the B3 clone not having CA activity are discussed in Chapter 4 of this dissertation. Resequencing of the Cah6 gene in the B3 clone needs to be done to check for any introduced mutations by the bacteria cells, before arriving at a conclusion about the activity of the purified Cah6 protein from B3 clone. If no mutations are found in the Cah6 sequence in the B3 clone, then site directed mutagenesis experiments are needed to alter the arginine residue in the enzymatically active Cah6 from B48 clone to study if the single arginine residue in Cah6 plays a role in modulating the catalytic activity of Cah6. The Cah6 protein has two cysteines and one histidine residue that can act as zinc coordinating ligands, as is seen in enzymatically active β-CAs. These conserved residues can be altered by site directed mutagenesis to verify their role in modulating the catalytic activity of Cah6.

Recently, a thorough search of the genomic and EST database of *C. reinhardtii* led to the identification of another putative β-CA gene. We have named it Cah7. Cah7 has only one EST record. Hence it is expressed in the cell but at low level. BLAST searches with Cah7 protein sequence showed that Cah7 has 28%-33% sequence identity with the mitochondrial Ca1 and the chloroplastic Cah6 protein from *C. reinhardtii*. It has 50% homology with the cytoplasmic β-CA protein from the green alga *Coccomyxa*. The 5' end of this gene needs to be clarified by rapid amplification of cDNA ends (RACE) before one can make any prediction about the localization of Cah7. Once the 5' end of Cah7 is resolved, it can be overexpressed in the pMal-c2x vector to study its biochemical properties and to raise an antibody against it, if the protein is enzymatically active. If Cah7 is found active, gene specific mutants can be generated using antisense or RNA interference technique to study its functional role, if any, in photosynthesis and CCM.

Activities of Ca1, Ca2, Cah3, Cah7 (cellular location still unknown) and Cah6 jointly constitute the total intracellular CA activity. Ca1 and Ca2 (mitochondrial CAs) have only one amino acid difference in the transit peptide. Hence the mature mitochondrial β-CAs (Ca1 and
Ca2) are identical in amino acid sequence. Ca1 has been cloned in the pMal-c2x vector to be expressed as a MBP-fusion protein (Eriksson et al., 1994). There is no report on the measurement of the CA activity in the purified recombinant fusion Ca1. In future experiments the recombinant Ca1 can be purified and the CA activity can be biochemically characterized. Cyanobacterial β-CA and the “soluble” chloroplast CA activity of C. reinhardtii have been shown to possess a high requirement for Mg$^{2+}$ (Price et al., 1992; Amoroso et al., 1996). The effect of MgSO$_4$, different CA inhibitors and thiol reducing agents, on the activities of soluble known CAs (Cah6, Cah7, Ca1 and Ca2) can be studied in future. The biochemical characteristics (particularly $I_{50}$ of different CA inhibitors and the effect of Mg$^{2+}$ on the CA activity) of Ca1/Ca2, Cah7, Cah3 and Cah6 can be exploited to selectively study the CA activity of each of the intracellular CAs in vivo.

Analyses of the expression patterns of CAs known to be involved in CCM like Cah3 and Cah1 in the Cah6 RNAi mutants by Northern blotting can be helpful in determining if there is a relative difference in the expression patterns of these CAs in the Cah6 mutants and the wild type cells. This experiment can give us some indication, if in the Cah6 RNAi mutants the expression of the CCM involved CAs, are upregulated to compensate for the underexpression of Cah6. Immunoblotting and Northern blotting analysis (using Cah3 and Cah6 specific probe) of cia5 and wild type cells of C. reinhardtii can show the expression patterns of the Cah3 and Cah6 genes in this mutant and can indicate if these genes are under the regulation of the Cia5 gene.

Maintenance of the HCO$_3^-$ pool in the stroma is essential for the operation of CCM. To prevent the dissipation of the HCO$_3^-$ pool, CA activity is absent in the cytosol of cyanobacteria. The expression of human CA in the cytoplasm of Synechococcus PCC7942 cells results in a massive leakage of CO$_2$ from the cells producing a HCR phenotype and implicating the carboxysomes as the site of CO$_2$ elevation (Price and Badger, 1989a). Analyses of signal peptide
seem to indicate that Gclp1 is located in the cytoplasm (Chapter 5). *Coccomyxa*, a green alga does not have a CCM (Palmqvist *et al.*, 1994). It has a cytoplasmic β-CA. It might be possible that the Gclp1 has become catalytically inactive in the course of evolution of CCM in *Chlamydomonas* or has some other enzymatic roles. Interrelationship between the presence or absence of CCM and cytoplasmic CAs in phylogenetically related green algae can be studied to arrive at an answer. Gclp1 has 40%-43% homology to some unknown proteins in rice, maize and *Arabidopsis* and also to ferripyochelin binding proteins in bacteria. Hence other physiological roles of Gclp1 cannot be ruled out.

Certain proteins that are toxic to bacteria can be overexpressed in yeast cells. Gclp1 can be overexpressed in eukaryotic yeast cells to check for CA activity. An antibody can be generated against the purified Gclp1 protein to immunolocalize Gclp1 in *Chlamydomonas* cells. This can verify its location in cytoplasm. The *Chlamydomonas* EST database has another putative γ-CA homologue, Gclp2 which is 80% similar to Gclp1. The *Arabidopsis* genome contains at least three homologues of γ-CA but it is not yet known if these gene products are active CAs or what physiological roles they might be playing in the cell. Gclp1 and Gclp2 have more than 40 ESTs and are highly expressed in cells. Northern blot analyses of these two genes using air and high CO$_2$ adapted cells can indicate if the expression of these genes is regulated by CO$_2$ concentration in the air. Since Gclp1 has similarities with ferripyochelin binding proteins, Northern blot analyses using cells grown in the presence and absence of iron in the nutrient medium, can indicate if iron concentration in the external medium can control the expression of these genes. In the future, generation of mutants of these putative γ-CA genes by antisense RNA or RNAi might be useful in probing the functional roles of these genes.

To our knowledge no other β-α- or γ-CA genes, other than those that have been already identified (Table 6.1), has been found in the EST and genomic database of *C. reinhardtii*. At this
point, *Arabidopsis* has five, six and three different genes that align well with the α-, β- and the γ-CAs, respectively. The availability of *Arabidopsis* and *Chlamydomonas* genome sequences and EST databases can be used to find out the exact number of expressed CA isoforms in these organisms. The challenge for future researchers will be to determine the expression patterns, localization and physiological roles for each of these isoforms. As there appears to be a large number of isoforms in plants and algae, CA researchers should be busy in the near future.
REFERENCES


Price G.D. and Badger M.R. (1989b) Isolation and characterization of high-CO$_2$ requiring mutants of the cyanobacterium Synechococcus PCC7942: two phenotypes that accumulate inorganic carbon but are apparently unable to generate CO$_2$ within the carboxysome. Plant Physiol. 91: 514-525.


The orange block arrows and interconnecting lines represent the exons and the introns, respectively. The 5’ end and the 3’ end primers are denoted by black and red arrows, respectively.

5’ end primers: - (5’ → 3’)

MP       AGCAACCGCAGCAGCCTT
SP2      ATGGGATGCGGTGCCAGCGTG
F4       GCACGAGGCAACATTTAAACA
F5       GCATCCCCACCCAGGACTTCA
F6       CCTGAGCAGGGTGGAATGGAA
X9       AAACTCAACTCTTCTACAATAGGC

3’ end primers: - (5’ → 3’)

R4       TTGCGCCATGAAGTCCCTAA
R6       TTGACGTTCTCCTCACCAC
R5       TGCGGTACAGATTACAGTCA

Cah6 (2885 bp)
The primer R4H was generated by adding a HindIII site (shown in blue) and four extra bases (shown in black) at the 5′ end of the primer R4.
The orange block arrows and interconnecting lines represent the exons and the introns, respectively. The 5′ end and the 3′ end primers are denoted by black and red arrows, respectively.

5′ end primers: - (5′ → 3′)

X1  TCCCCCGGGCAGATGATACCAGCAACATTAAACA
X3  CGGGATCCCGTGAGCGAATTGATCTTTTCA
X5  TCCCCCGGAAAACTCAACTCCTTCATAATAGGC
X7  CGGGATCCCCTCGCAGATACATCCCT
3’ end primers: - (5’ → 3’)

X2        CGGAATTCCGTGAGCGAATTGATCTTTTCA
X4        GCTCTAGACAGATGATACCAGCAACATTAAACA
X6        CGGAATTCCCTCGC AGA TAC ATC CCT
X8        GCTCTAGAAAAACTCAACTCCTTCATAATAGGC
APPENDIX 3

Gclp1 MAP AND PRIMERS

The orange block arrows and lines interconnecting the block arrows represent the exons and introns, respectively. 5' end primers and 3' end primers are shown by black and red arrows, respectively. The broken line in B1 primer denotes the missing DNA sequence in the primer.

5' end primers: - (5' → 3')

M1F  GCAGTTAGTCCATGCGAA  
F1  CCGCAAGCCGGATGTCGCCGTTAA  
F2  GGGGGCGGGCCTCATTGCTGTGTTATG  
FS1  GCTTTCTACGTGACACAAGG  
FS2  GGACCATGTCGCTATTCA  
F4  GCTGCTACGCCGAGCTGTC  
S1  ATGTCGCTATTCAAGTCTAGCCTG  
S3  GCGCTGGATGAGCTGGGC  
F3  CCTTGTGCGGGGCTTTCTAC
3’ end primers: - (5’ → 3’)

M1R   TTGAATGAGTACGGTGCA
BS1   GTAAAGAGAGGAGCTGCAAG
BS2   GCCAAGAGGTAAAGAGAGGA
B1    CAATGTGACCATTGGGACGACGCGC
B2    ACACCCACATCCCTCGGAGCAG
B6    TGTCCAAAGTACAGCATGG
M1RH  ATATGAAGCTTTGAATGAGTACGGTGCA

FS1rev, FS2 rev, F1 rev, F2rev, B4, B6rev, M1F rev, M1R rev, B1rev, B2rev, B4 are reverse complementary primers of FS1, FS2, F1, F2, F4, B6, M1F, M1R, B1, B2 and F4, respectively. The primer R4H was generated by adding a HindIII site (shown in blue) and four extra bases (shown in black) at the 5’ end of the primer R4.
VITA

Mautusi Mitra was born in Calcutta, India, on February 14, 1968. She attended Presidency College at Calcutta, India, majoring in botany. She went on to complete her Bachelor of Science degree in botany at the University of Calcutta, India, in 1989. She pursued a Master of Science degree in botany at the University of Calcutta, India, which she received in 1991. After receiving her master’s degree, she pursued personnel management for five years. In 1996, Mautusi received a Junior Research Fellowship from the Council of Scientific and Industrial research in India. She then attended the Graduate School at Louisiana State University to pursue a doctoral degree in plant biology. Her dissertation research has dealt with the identification, cloning and overexpression of carbonic anhydrase and carbonic anhydrase like genes of the green alga *Chlamydomonas reinhardii*, under the supervision of Dr. J.V. Moroney. After her graduation, Mautusi, is going to join Dr. Anastasios Melis’s laboratory at the University of California, Berkeley, where she will be doing research on photosystem II repair mechanisms and hydrogen production in green algae.