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Intracellular Carbonic Anhydrase of *Chlamydomonas reinhardtii*[1]

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An intracellular carbonic anhydrase (CA; EC 4.2.1.1) was purified to homogeneity from a mutant strain of *Chlamydomonas reinhardtii* (CW 92) lacking a cell wall. Intact cells were washed to remove periplasmic CA and were lysed and fractionated into soluble and membrane fractions by sedimentation. All of the CA activity sedimented with the membrane fraction and was dissociated by treatment with a buffer containing 200 mM KCl. Solubilized proteins were fractionated by ammonium sulfate precipitation, anionic exchange chromatography, and hydrophobic interaction chromatography. The resulting fraction had a specific activity of 1260 Wilbur-Anderson units/mg protein and was inhibited by acetazolamide (50% inhibition concentration, 12 nM). Final purification was accomplished by the specific absorption of the enzyme to a Centricon-10 microconcentrator filter. A single, 29.5-kD polypeptide was eluted from the filter with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and a 1.5 M ammonium sulfate eluate contained CA activity. In comparison with human CA isoenzyme II, the N-terminal and internal amino acid sequences from the 29.5-kD polypeptide were 40% identical with the N-terminal region and 67% identical with an internal conserved region. Based on this evidence, we postulate that the 29.5-kD polypeptide is an internal CA in *C. reinhardtii* and that the enzyme is closely related to the α-type CAs observed in animal species.

The eukaryotic unicellular green alga *Chlamydomonas reinhardtii*, as well as many other green algae and cyanobacteria, has the ability to regulate its internal level of C_i in response to varying external levels of C_i during growth. When *C. reinhardtii* are grown in a high-C_i environment (1–5% CO_2 in air), the diffusion of C_i into the chloroplast is sufficient for efficient photosynthesis to occur. However, when cells are grown under low external C_i levels (air grown), a CCM is induced (Aizawa and Miyachi, 1986; Badger and Price, 1992, 1994).

One component of this CCM is a low-C_i-induced pCA (EC 4.2.1.1, carbonate lyase, carbonate dehydratase) (Coleman and Grossman, 1984; Yang et al., 1985). CA is a zinc-containing metalloenzyme that catalyzes the interconversion of CO_2 and HCO_3^-. The proposed role for the pCA is to ensure that chemical equilibrium always exists outside the plasma membrane between the predominant C_i species at pH values near neutrality, HCO_3^- and CO_2 (Moroney et al., 1985), allowing CO_2 to enter the cell at a sufficient rate to support photosynthesis.

Another pCA that is expressed under high C_i was isolated by Rawat and Moroney (1991). The low-C_i-inducible pCA (product of the CAH1 gene) and the high-C_i-expressed pCA (product of the CAH2 gene) (Tachiki et al., 1992) are 92% homologous and show significant homology to the α-type CAs (Fukuzawa et al., 1990) that are found predominantly in mammals and are classified based on their sharing of a significant degree of sequence homology (Venta et al., 1987; Tashian, 1992). A distinct β type of CA that shares no sequence homology to α type is present in *Escherichia coli* (Sung and Fuchs, 1988; Guilloton et al., 1992) and *Synechococcus* (Fukuzawa et al., 1992) and in higher plant chloroplasts (Burnell et al., 1990; Fawcett et al., 1990; Roeske and Ogren, 1990; Majeau and Coleman, 1991, 1992; Raines et al., 1992) and has recently been found in the green alga *Coccomyxa sp.* (Hiltonen et al., 1995). The α-type CAs are typically monomeric with an apparent molecular mass of about 30 kD, whereas the chloroplastic β-type CAs are found to be hexameric with a subunit mass of 26 to 34 kD (Reed and Graham, 1981). The two types of CAs also differ in their sensitivities to inhibition by sulfonamides. Several physiological roles have been proposed for the higher plant CAs, including the importance of supplying substrate, CO_2, to Rubisco in the alkaline stroma (Reed and Graham, 1981; Raven and Newman, 1994).

It has further been postulated that intracellular CAs are present in algal cells, located in the chloroplast and/or in the cytoplasm (Spalding et al., 1983; Sültemeyer et al., 1990; Moroney and Mason, 1991). Chloroplasts from low-C_i-grown *C. reinhardtii* were shown to contain higher CA activity compared to chloroplasts from high-C_i-grown cells (Sültemeyer et al., 1990; Karlsson et al., 1994; Katzman et al., 1994; Ramazanov and Cárdenas, 1994). Husic and Mar-

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Abbreviations: AZA, acetazolamide; CA, carbonic anhydrase; CCM, carbon dioxide-concentrating mechanism; C_i, inorganic carbon; HIC, hydrophobic interaction chromatography; IEC, anion-exchange chromatography; pCA; periplasmic carbonic anhydrase; WAU, Wilbur-Anderson units.

cus (1994) identified a polypeptide with an apparent molecular mass of 30 kD as being an intracellular CA using a CA-directed photoaffinity label. In this report we present evidence for the isolation and characterization of an intracellular CA of the α type from *C. reinhardtii*.

MATERIALS AND METHODS

Culture Conditions

The cell-wall-less mutant of *Chlamydomonas reinhardtii*, CW 92, was cultivated in continuous light at an incident PPFD of 150 μmol photons m^{-2} s^{-1}, obtained from fluorescent tubes (TL 40W/55; Philips, Eindhoven, The Netherlands) in a phosphate-buffered medium at pH 7.5 (Surzycki, 1971; Solter and Gibor, 1977). The cultures were grown in 5-L glass bottles at 28°C and were supplied with 5% CO_2 in air. Twelve hours prior to harvesting, cultures were subjected to vigorous bubbling with air.

Measurement of CA Activity

CA activity was electrochemically determined by measuring the time for the pH to decrease from 8.0 to 7.2, at 2°C, in a sample of 4 mL of 20 mM veronal buffer, pH 8.3, upon addition of 2 mL of ice-cold CO_2-saturated distilled H_2O. One WAU (Wilbur and Anderson, 1948; Yang et al., 1985) of activity was defined as: WAU = $(t_0 - t)/t$, where t_0 was the time for the pH change with buffer controls and t was the time obtained when CA-containing samples were added. To determine the AZA inhibition of CA, activity was measured in partially purified samples from both high- and low-C_i-adapted cells as described above, but with additions of nanomolar concentrations of AZA.

Determination of Chl and Protein

Chl concentration was determined spectrophotometrically by the method of Porra et al. (1989), and the concentration of protein was determined by the method of Bradford (1976), using a UV/visible spectrophotometer (UV-120–02; Shimadzu, Kyoto, Japan). BSA was used as a protein standard.

Isolation of CA

All purification steps were performed at 4°C. Thirty liters of *C. reinhardtii* cells in the logarithmic growth phase were harvested by centrifugation (700*g*, 10 min) and washed once in 25 mM Tris-HCl buffer, pH 8.0 (buffer A). The cells were washed free of pCA for 15 min in buffer A containing 200 mM KCl (Husic and Quigley, 1990) and then washed two additional times in buffer A. The pellet was resuspended in buffer A, and the intact cells were assayed for CA activity to verify that pCA activity had been washed from the cells. The cells were homogenized in a precooled French press cell (Aminco, Silver Spring, MD) at 160 MPa, and the CA activity of the lysate was measured to assay for internal CA activity. The homogenate was centrifuged at 100,000*g* for 2 h, and the supernatant (supernatant I) and pellet were assayed for CA activity to determine the distribution of CA between the soluble and insoluble phases. More than 95% of the activity was typically found in the insoluble fraction. To solubilize CA, the pellet was resuspended in 12.5 mM Tris-HCl buffer, pH 8.2, and 1 μM $ZnSO_4$ (buffer B), containing 200 mM KCl. The suspension was gently stirred on ice for 15 min, followed by a second ultracentrifugation at 100,000*g* for 1 h. After determination of CA activity, the supernatant (supernatant II) was subjected to $(NH_4)_2SO_2$ fractionation. The proteins precipitated between 35 and 65% of saturation were pelleted by centrifugation at 50,000*g* for 30 min and resuspended in 7.5 mL of buffer B. $(NH_4)_2SO_4$ was removed by desalting the sample using PD10 columns (Pharmacia) equilibrated with buffer B. The desalted sample (7.6 mL) was applied to an IEC column (1.6 × 15 cm, Q Sepharose Fast Flow; Pharmacia) equilibrated with buffer B. The column was washed with the equilibration buffer until the A_{280} of the eluate was close to zero and then washed with about 5 volumes of buffer B containing 100 mM KCl. CA activity was eluted with 125 mM KCl in buffer B, and no additional CA activity was eluted by subsequent washes with buffer B containing either 150 or 200 mM KCl.

Fractions with CA activity were pooled (116 mL) and the buffer concentration was increased to 50 mM Tris-HCl, pH 8.2, and 1 μM $ZnSO_4$ (buffer C) by adding concentrated buffer from a stock solution. $(NH_4)_2SO_4$ was added to the sample to a final concentration of 1.5 M, and the sample was applied to an HIC column (0.9 × 4 cm, phenyl-Sepharose 6FF, High Substitution; Pharmacia), which previously had been equilibrated with buffer C containing 1.5 M $(NH_4)_2SO_4$. The column was sequentially washed with 4 volumes of buffer C containing 1.5 M $(NH_4)_2SO_4$ followed by buffer C containing 1.0 M $(NH_4)_2SO_4$. CA activity was eluted with buffer C containing 0.85 M $(NH_4)_2SO_4$. Fractions with CA activity were pooled (8.5 mL), precipitated with 65% $(NH_4)_2SO_4$, pelleted by centrifugation at 50,000*g* for 30 min, and resuspended in 0.8 mL of buffer B. This sample was used for analysis of specific activity, inhibition by AZA, and immunological studies.

The final purification step was performed by dividing and diluting the sample 5-fold by addition of buffer B to 2 mL each, followed by concentration with Centricon-10 microconcentrators (Amicon, Danvers, MA) to 100 μL, and the procedure was repeated once. This treatment caused an almost complete loss of CA activity in both the retentate and the eluate. The Centricon-10 filter surfaces were rinsed with either SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) or buffer B containing 1.5 M $(NH_4)_2SO_4$, and the rinsing solutions were collected. SDS-PAGE (12.5% polyacrylamide) (Laemmli, 1970) with Coomassie brilliant blue R-250 staining revealed that the eluate contained a single polypeptide of 29.5 kD. The eluate from $(NH_4)_2SO_4$-treated microconcentrator filters contained CA activity.

Immunoblotting

The partially purified sample after the HIC step was subjected to SDS-PAGE (12.5% polyacrylamide) and transferred electrophoretically to a nitrocellulose filter (0.2-μm

Table I. *Purification of intracellular CA from C. reinhardtii*

Purification Step	Total Activity	Total Protein	Specific Activity	Yield	Purification
	WAU	*mg*	*WAU/mg protein*	*%*	*-fold*
Homogenate	1330	2400	0.552	100	1.00
Supernatant I	79.6	1305	0.061	6.00	0.11
Supernatant II	1190	824	1.44	89.0	2.61
Precipitation in 35–65% $(NH_4)_2SO_4$	1130	190	5.97	85.3	10.8
IEC	441	1.74	253	33.1	458
HIC	180	0.085	2040	13.5	3700
Precipitated	94.8	0.075	1260	7.13	2280

pore size, Bio-Rad) for western blot analysis using antisera directed against pCA from *C. reinhardtii* or pea chloroplastic CA. Horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence (Amersham) were used for detection of the antibody-antigen conjugate.

Amino Acid Sequencing

The N-terminal sequence of the purified CA was determined following SDS-PAGE (12.5% polyacrylamide) and electrophoretic transfer to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was stained with Coomassie brilliant blue in 20% methanol and 0.5% acetic acid to visualize the 29.5-kD polypeptide, which was subjected to N-terminal sequence analysis in an Applied Biosystems model 476A sequencing system.

Internal amino acid sequences were determined following SDS-PAGE (12.5% polyacrylamide) of the purified CA and staining of the gel as above. The 29.5-kD band was excised and subjected to digestion with trypsin (modified trypsin, Promega) according to the method of Rosenfeld et al. (1992). The resulting peptides were separated on a Pharmacia SMART chromatography system with a μRPC C2/C18 SC 2.1/10 column. Buffers used in the separation were solution A, 0.1% TFA in H_2O, and solution B, 90% acetonitrile and 0.1% TFA in H_2O. A gradient from 0 to 50% of solution B (in solution A) in 75 min was run and followed by a gradient of 50 to 100% B in 5 min. The collected peptides were subjected to amino acid sequence analysis.

RESULTS

Isolation of CA

The results of the steps used for the purification of *C. reinhardtii* intracellular CA are summarized in Table I. The initial steps included salt washes (0.2 M KCl) of intact cell-wall-deficient cells to remove pCA, the fractionation of soluble and insoluble proteins by sedimentation, and the dissociation of the intracellular CA by a salt wash of the particulate fraction that contained more than 95% of the CA activity in cell lysates. The resulting CA-containing fraction was subsequently subjected to IEC and HIC, resulting in a 2300-fold purification of the intracellular CA. To obtain a homogenous preparation of the enzyme, the CA was specifically absorbed and eluted from Centricon-10 microconcentrator filters, resulting in a single, 29.5-kD polypeptide following SDS-PAGE (Fig. 1).

AZA Inhibition of CA Activity

When the CA preparation obtained following HIC was used, 50% inhibition of CA activity was observed with 12 nM AZA.The inhibition pattern of partially purified CA from high-C_i-grown cells was similar (Fig. 2). The low concentration of AZA that resulted in a 50% inhibition of the intracellular CA activity in *C. reinhardtii* is similar to values for the inhibition of α-type CAs, 2 to 200 nM, and different from the less sulfonamide-sensitive β-type CAs, 5 to 500 μM (Sültemeyer et al., 1993).

Immunoblotting

Immunoblots of the purified internal CA using antibodies directed against either the pCA from *C. reinhardtii* or pea chloroplastic CA showed no cross-reaction to the

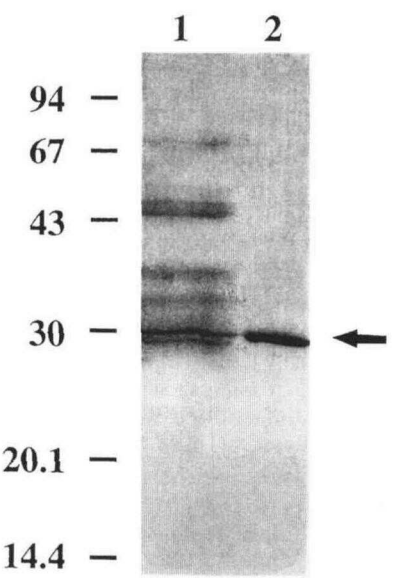

Figure 1. SDS-PAGE analysis of purified intracellular CA of *C. reinhardtii*. Lane 1, Retentate after concentrating the final sample on a Centricon-10 microconcentrator; lane 2, sample rinsed from the Centricon-10 microconcentrator filter surface with SDS-PAGE sample buffer. The arrow denotes *C. reinhardtii* intracellular CA. Molecular mass standards in kD are indicated on the left.

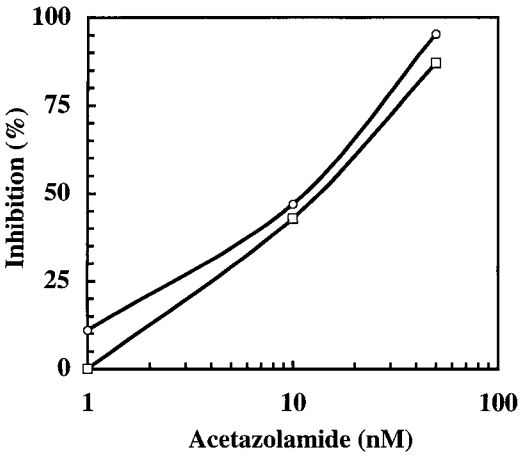

Figure 2. AZA inhibition of CA activity in high(□) and low (○) C_i-grown cells.

29.5-kD polypeptide (data not shown), indicating that the isolated intracellular CA is immunologically distinct from pCA and plant chloroplast CA.

Amino Acid Sequencing

Amino acid sequencing of the intact 29.5-kD polypeptide resulted in a 15-residue amino-terminal sequence, and sequence analysis of an internal tryptic peptide resulted in a 20-residue sequence. The sequences were aligned with human CAs (isoenzymes I-VI) and the sequences of the two *C. reinhardtii* pCAs (Fig. 3) obtained from the SwissProt data base using the Wisconsin Sequence Analysis Package, version 8.0 (Genetics Computer Group, Madison, WI). The alignment confirms that the *C. reinhardtii* 29.5-kD polypeptide amino acid sequences are derived from a CA of the α type. The internal fragment sequence of the isolated *C. reinhardtii* internal CA was 67% identical with the conserved region of human CAII (residues 112–124), and the N termini of *C. reinhardtii* internal CA and human CAII were 40% identical. The conservation of His residues possibly liganded to the active site zinc ion and residues forming the hydrogen-bound network to zinc-bound solvent molecules provide further evidence that the sequence is derived from an α-type CA.

To verify that the polypeptide migrating at 35 kD was not contaminating degraded pCA, its N terminus was also sequenced. The resulting sequence, ATKASTAVTTDMSKR, was confirmed to be identical with that of a region 35 amino acids downstream of the N terminus of the translated cDNA clone encoding the *C. reinhardtii* Fd-$NADP^+$ reductase (Kitayama et al., 1994), possibly indicating a processing site of the chloroplast-targeted precursor.

DISCUSSION

In this report we have documented the isolation of a 29.5-kD polypeptide that is an intracellular CA from *C. reinhardtii*. Several results are consistent with the isolation of an intracellular CA distinct from the previously identified pCAs from this species. The activity was observed only after lysis of salt-washed wall-less cells. The isolated protein had an apparent molecular weight and partial amino acid sequences that differed from the documented periplasmic forms of the CA, and prior to a final purification step, the preparation had a high catalytic specific activity for CO_2 hydration. Furthermore, N-terminal and internal amino acid sequence analysis revealed a high degree of sequence homology with CAs of the α type, which is characteristic of the enzymes from mammalian sources. The apparent molecular mass of 29.5 kD and the association of the CA with an insoluble fraction upon cell lysis are also characteristics observed by Husic and Marcus (1994) for a 30-kD polypeptide in *C. reinhardtii* that was believed to represent intracellular CA based on specific labeling in cell extracts with a CA-directed photoaffinity reagent. No such labeling was observed in *C. reinhardtii* mutant lines believed to be deficient in intracellular CA.

It has been suggested that *C. reinhardtii* and other green microalgae with a CCM have isoenzymes of intracellular CA in both the cytosol and the chloroplast (Spalding et al.,

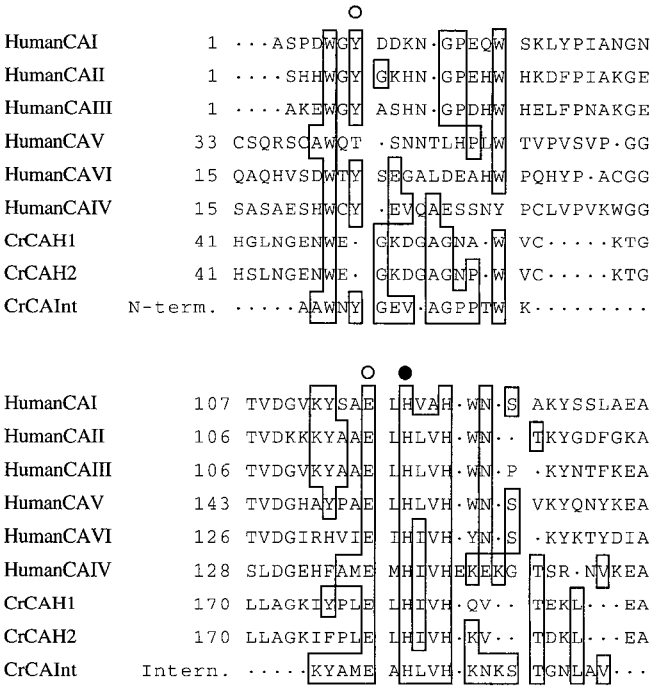

Figure 3. Alignment of the partial N-terminal and internal amino acid sequence from the *C. reinhardtii* internal CA with the corresponding sequences for known α-type CAs. The SwissProt data base accession numbers for human CAI to CAVI and *C. reinhardtii* CAH1 and CAH2 sequences are P00915, P00918, P07451, P22748, P35218, P23280, P20507, and P24258, respectively. The numbers indicate the amino acid position in the given sequences. Boxed residues indicate that the *C. reinhardtii* internal CA sequences are similar to one or more of the aligned sequences in these positions, and dots indicate where gaps are inserted to obtain optimal matching. Putative zinc-liganded His residues and residues forming the hydrogen-bound network to zinc-bound solvent molecules are depicted by ● and ○, respectively.

1983; Sültemeyer et al., 1990; Moroney and Mason, 1991). However, our results did not provide evidence for more than one isozymic form of intracellular CA. The studies providing evidence for several intracellular isozymes were based on results from a mass spectrometric technique, which may have detected CAs not readily apparent using the pH change method that was used in our experiments. However, it is possible that there is another form of CA that is inactivated on cell lysis or that has very low relative activity and is lost upon purification.

When the homogenate from *C. reinhardtii* cells was centrifuged at 100,000*g* for 2 h to pellet cell walls, membranes, membrane vesicles, and other particulate matter, nearly all of the CA activity was found in the pelleted fraction. In our hands, an extensive centrifugation is necessary to completely separate the soluble and insoluble proteins, since less rigorous centrifugation leaves some of the lighter thylakoid membranes in the supernatant, as judged by the distribution of Chl. Thus, our results show that a major proportion of the internal CA is in a particulate fraction. Protease inhibitors were not included in the breaking buffer, and therefore, we cannot exclude the partial proteolysis and inactivation of a soluble CA upon purification. However, to minimize the possibility of proteolysis, the time from cell lysis to activity measurement was always only a few minutes when the sample was kept on ice. In experiments in which a lower speed was used for centrifugation, and some activity remained in the supernatant, it took at least 1 h before the CA activity in the supernatant was no longer detectable. After the salt treatment of the pellet to release CA from the particulate fraction, the activity was more stable. The finding that salt treatment of the high-speed pellet solubilized CA activity indicates that the intracellular CA could be peripherally associated with membranes, charge screened by the salt, or that it is associated with a particulate fraction, possibly in the pyrenoids that may be disrupted and dissociated by the salt. Kuchitsu et al. (1991) showed that the pyrenoid bodies from low-C_i-grown *C. reinhardtii* cells are stained in situ by the fluorescent CA inhibitor dansylamide, thus indicating one possible location of intracellular CA.

The binding of the CA to the Centricon-10 filter was unexpected and allowed for the specific absorption of the internal CA as a final step in the purification of the 29.5-kD polypeptide. Some of the human isozymes of CA possess similar adsorption properties (B.-H. Jonsson, Umeå University, personal communication). According to the manufacturers of the filters, adsorption of proteins to the filter surface and support materials are dependent on their hydrophobicity, the concentration of the solute, sample composition, and pH.

We observed it to be crucial that the AZA inhibition of CA was carried out on a purified sample, since in homogenates the CA appeared less sensitive to AZA inhibition. Since sensitivity to sulfonamide inhibitors is one characteristic used to classify CAs, the determination of sulfonamide inhibition characteristics with crude cell extracts may yield misleading interpretations of the CA class based solely on sulfonamide inhibition data. Furthermore, this observation may explain reports that the intracellular CA in *C. reinhardtii* is relatively insensitive to inhibition by sulfonamides (Husic et al., 1989; Sültemeyer et al., 1990), when compared to the enzyme isolated in this report. Although the reason for this phenomenon is not clear, possible explanations include the binding of other proteins to the CA or AZA or the insolubility of the CA in the cell extracts, possibly limiting the accessibility of the enzyme to inhibitors.

Immunoblot analysis of the purified intracellular CA with antibodies raised against the *C. reinhardtii* 37-kD pCA showed no specific cross-reaction, even though they seem closely related on an amino acid sequence basis (Fig. 3). This indicates that intracellular CA and pCA from *C. reinhardtii* are less similar than are the two different isozymic forms of pCA, since antibodies raised against the low-C_i-inducible CA cross-react with the high-C_i-expressed pCA (Rawat and Moroney, 1991).

Antibodies raised against a synthetic peptide corresponding to the 13 amino acids of the N terminus of the internal CA showed immunoreactivity with the 29.5-kD polypeptide but not to pCA (data not shown). It was also determined that antibodies raised against higher plant chloroplastic CA did not cross-react with the *C. reinhardtii* internal CA. These results are consistent with earlier reports that the α- and β-type CAs are completely different at the amino acid sequence level.

N-terminal and internal amino acid sequencing of the purified protein revealed a high degree of similarity with known α-type CAs, including the conservation of His residues that are liganded to the active site zinc ion and residues forming the hydrogen-bound network to zinc-bound solvent molecules. From these results, we conclude that the internal CA that we have isolated from *C. reinhardtii* is an α-type CA.

Prior to this report, the only reports of the isolation of internal CA from unicellular algae were from the red alga *Porphyridium cruentum* (Yagawa et al., 1987a) and the green alga *Coccomyxa* (Hiltonen et al., 1995). The reported native molecular mass of the red algal CA was 55 to 59 kD, as a monomer. Immunogold labeling of *P. cruentum* with antibodies directed against its CA suggests that it is primarily localized in the chloroplast but was also observed to some extent in the cytoplasm (Yagawa et al., 1987b). Inhibition studies showed that it was fairly sensitive to AZA, as are the α-type CAs. The *Coccomyxa* CA was reported to have a native molecular mass of 80 kD, as a trimer, with a subunit size of 26 kD (Hiltonen et al., 1995). It was confirmed to be a β-type CA by internal amino acid sequences and also by inhibition studies. Further studies of the CA isolated in this report will address structural characteristics in the *C. reinhardtii* intracellular CA.

In this report we have described the identification, isolation, and initial characterization of a novel intracellular CA in *C. reinhardtii* that is believed to play an important role in CO_2 assimilation. Further studies of its structure, localization, and regulation of catalytic activity and synthesis will be critical to the elucidation of the functional and structural details of the CCM in this species.

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