Intracellular β-Carbonic Anhydrase of the Unicellular Green Alga Coccomyxa

Cloning of the cDNA and Characterization of the Functional Enzyme Overexpressed in Escherichia coli

Thomas Hiltonen, Harry Björkbacka, Cecilia Forsman, Adrian K. Clarke, and Göran Samuelsson

Department of Plant Physiology (T.H., A.K.C., G.S.), and Department of Biochemistry (H.B., C.F.), University of Umeå, 901 87 Umeå, Sweden

Carbonic anhydrase (CA) (EC 4.2.1.1) enzymes catalyze the reversible hydration of CO₂, a reaction that is important in many physiological processes. We have cloned and sequenced a full-length cDNA encoding an intracellular β-CA from the unicellular green alga Coccomyxa. Nucleotide sequence data show that the isolated cDNA contains an open reading frame encoding a polypeptide of 227 amino acids. The predicted polypeptide is similar to β-type CAs from Escherichia coli and higher plants, with an identity of 26% to 30%. The Coccomyxa cDNA was overexpressed in E. coli, and the enzyme was purified and biochemically characterized. The mature protein is a homotetramer with an estimated molecular mass of 100 kDa. The CO₂-hydration activity of the Coccomyxa enzyme is comparable with that of the pea homolog. However, the activity of Coccomyxa CA is largely insensitive to oxidative conditions, in contrast to similar enzymes from most higher plants. Fractionation studies further showed that Coccomyxa CA is extrachloroplastic.

CA (EC 4.2.1.1) is a zinc-containing enzyme that catalyzes the reversible reaction CO₂ + H₂O ↔ HCO₃⁻ + H⁺. CA is widely distributed throughout nature, from eukaryotes such as vertebrates, invertebrates, and plants, to prokaryotes such as archaeabacteria and eubacteria. The enzyme is classified into three independent CA gene families designated α, β, and γ (Hewett-Emmett and Tashian, 1996). The α-CAs are found primarily in animals (Tashian, 1992), but homologs have also been identified in the bacterium Neisseria gonorrhoeae (Hewett-Emmett and Tashian, 1996) and the green alga Chlamydomonas reinhardtii (Fukuzawa et al., 1990). This is the most extensively studied CA family, and includes the biochemically well-characterized mammalian CA isozymes, the crystal structures of which have been solved to high resolution (Kannan et al., 1975; Eriksson et al., 1988; Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995).

Conversely, the γ-CAs are a newly discovered gene family, with the enzyme from Methanosarcina thermophila being the only γ-CA isolated and characterized thus far (Alber and Ferry, 1994). Related sequences have been found in several eubacteria and in Arabidopsis (Hewett-Emmett and Tashian, 1996), but it is not known as yet whether they encode functional CAs. The crystal structure for the γ-CA from M. thermophila is different from that of the α-type enzymes. The γ-CA is trimeric, with the active site situated between the subunits (Kisker et al., 1996).

CAs belonging to the β-CA family have been found in both C₃ and C₄ monocot and dicot plants, in the mitochondria of C. reinhardtii, and in various eubacteria (Eriksson et al., 1996; Hewett-Emmett and Tashian, 1996). Among the dicot species, the sequence similarity between the different β-CAs is around 80% (60% identity). The homology is slightly lower between the monocot and dicot homologs, but the similarity remains considerable (>70%). In contrast, the β-CAs found in prokaryotes are more variable and exhibit low sequence similarity to the plant homologs (30%). Alignment of all known amino acid sequences from functional β-CAs reveals invariant amino acid residues at 26 positions, of which most are found within two regions. Extended radiographic-absorption fine-structure analysis of spinach CA suggests a Cys-His-Cys-H₂O ligand scheme for binding of the zinc ion (Bracey et al., 1994; Rowlett et al., 1994). The first of these invariant Cys residues is found in one conserved region, whereas the His and the other Cys residue are situated in a second conserved region. However, to our knowledge, no three-dimensional structure has been described for any β-CA. Most of the biochemical studies have been done on chloroplastic homologs from C₃ dicots and on the Escherichia coli enzyme.

Chloroplastic β-CA is nuclear encoded and synthesized in the cytoplasm with an N-terminal transit peptide that targets the precursor into the chloroplast stroma (Forsman and Pilon, 1995). Subsequent maturation involves removal of the transit peptide, folding, and oligomerization. The native molecular masses of CAs from C₃ dicot plants have

Abbreviations: CA, carbonic anhydrase; CCM, inorganic carbon-concentrating mechanism; CD, circular dichroism; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); MBP, maltose-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEP, PEP carboxylase.
been reported to vary between 140 and 250 kD, with a subunit mass of 26 to 34 kD, each binding one zinc ion (Reed and Graham, 1981). The β-CA multimeric complex has been shown to consist of eight subunits (Aliev et al., 1986; Björkbacka et al., 1997). CAs from monocot plants have a monomeric mass of around 25 kD and an estimated native mass of 42 kD (Atkins et al., 1972; Atkins, 1974). The CA from E. coli is also reported to be an oligomer, most likely a tetramer or a dimer, depending on the experimental conditions (Guilleton et al., 1992).

The kinetic characteristics of chloroplast β-CA have been studied for both pea (Johansson and Forsman, 1993) and spinach enzymes (Pocker and Ng, 1973; Rowlett et al., 1994). Both possess a high catalytic efficiency, with $K_{cat}$ values between $10^9$ and $10^6$ s⁻¹ at high pH. The kinetic mechanism for these enzymes is consistent with the general mechanism proposed for the high-activity α-CA isozymes (Silverman and Lindskog, 1988).

The rate of the uncatalyzed interconversion between the two inorganic carbon species, CO₂ and HCO₃⁻, is insufficient to cope with the metabolic demand within a plant cell, but this varies among different organisms (for review, see Badger and Price, 1994). Because CA catalyzes the reversible hydration of CO₂, it has been proposed that chloroplastic CA is involved in the fixation of CO₂ in the Calvin cycle, which involves the carboxylating enzyme Rubisco (Reed and Graham, 1981). CO₂ is the only substrate for Rubisco, but HCO₃⁻ is the predominant carbon species within the alkaline chloroplast stroma (Makino et al., 1992). In addition, CA appears to play a role in the CCM present in certain cyanobacteria and free-living algae (for review, see Badger, 1987).

Some species of green algae and cyanobacteria that are photosynthetic components of lichens also possess CCMs, but there are some species that appear to lack this mechanism. The green alga Coccomyxa is one such photobiont (Palmqvist et al., 1994). The Coccomyxa genus is composed of many species forming symbiotic relationships in a small but diverse group of lichens (Honegger, 1991). This alga is unusual in that it lacks a CCM but has a very high intracellular CA activity (Hiltonen et al., 1995). The specific biological function of this internal CA in Coccomyxa is unknown at present, as is its subcellular location.

The aim of this study was to characterize the major intracellular β-CA from Coccomyxa. By cloning and sequencing the corresponding cDNA, we were able to overexpress the enzyme in E. coli and purify it to homogeneity. Structural and kinetic studies of this new β-CA demonstrated that the Coccomyxa enzyme possesses several interesting properties distinct from the higher-plant β-CAs. Furthermore, we demonstrated that the Coccomyxa CA is extrachloroplastic and probably located to the cytosol.

**MATERIALS AND METHODS**

**Determination of Internal Amino Acid Sequences**

Internal amino acid sequences were determined after SDS-PAGE (12.5% polyacrylamide) of previously semipurified Coccomyxa CA (Hiltonen et al., 1995). The gel was stained with 0.5% Coomassie brilliant blue in 20% methanol and 0.5% acetic acid to visualize the 25-kD polypeptide. The band was excised and subjected to digestion with modified trypsin (Promega) according to the method of Rosenfeld et al. (1992). The collected peptides were subjected to amino acid sequence analysis using a sequencing system (model 476A, Applied Biosystems).

**RNA Isolation and cDNA Library Construction**

Total RNA was isolated from 2-L Coccomyxa cultures grown according to the method of Hiltonen et al. (1995). Cells were centrifuged at 1,100g for 10 min at 4°C and the pellet (3.7 g) was resuspended in 10 mL of 50 mm Tris-HCl, pH 7.6, and 10 mm EDTA. Cells were lysed in a precooled French pressure cell (Aminco, Silver Spring, MD) at 160 MPa and immediately mixed with 40 mL of 4 mm guanidinium isothiocyanate, 50 mm Tris-HCl, pH 7.6, 10 mm EDTA, and 2% sarcosyl. The mixture was centrifuged at 4,000g for 5 min at 4°C. CsCl was added to the supernatant to a final concentration of 40% ($v/v$), and the supernatant was centrifuged for 10 min at 31,000g at 4°C. The resulting supernatant was laid on a 5.7 CsCl cushion and centrifuged for 18 h at 150,000g at 20°C. The pellet was resuspended in 200 μL of prewarmed (56°C) RNA-elution buffer (mRNA isolation kit, Stratagene) containing 1% SDS. The RNA sample was phenol extracted once and precipitated with ethanol, then the pellet was resuspended in 100 μL of elution buffer. Polyadenylated RNA was isolated from total RNA using an mRNA isolation kit. A Coccomyxa cDNA library was made from 5 μg of the purified poly(A⁺) RNA using a cDNA-synthesis kit (ZAP Express, Stratagene).

**Screening of the cDNA Library**

About 1.5 × 10⁵ plaque-forming units were spread among Escherichia coli XL1-Blue MRF (Stratagene) host cells on agar plates and analyzed according to standard procedures (Sambrook et al., 1989) using a 550-bp DNA probe corresponding to the 3′ end of the Coccomyxa CA. The probe was generated by PCR amplification from the Coccomyxa cDNA library using the degenerate primer 5′-CGGGAATTCGCGGGTAAAATATATTAAATATGAAGATAGC-3′, corresponding to the internal peptide sequence TAGVTNLWI, and the nondegenerate primer T₇ (22-mer, Perkin-Elmer) using 1 × 10⁵ plaque-forming units of the cDNA library. Hybridization to the radiolabeled probe was carried out at 65°C for 15 h in 4 × SSPE (1 × SSPE = 150 mm NaCl, 1 mm EDTA, and 15 mm sodium phosphate, pH 7.4), 5% Denhardt’s solution (0.05% Ficoll 400, 0.05% PVP, and 0.05% BSA), 0.5% SDS, and denatured salmon-sperm DNA (100 μg mL⁻¹). After hybridization the filters were washed at 65°C in 2 × SSPE, 0.5% SDS followed by 1 × SSPE, 0.1% SDS. Positive plaques were identified and isolated according to the instructions of the manufacturer (Stratagene).

**DNA Sequencing**

Selected clones were sequenced by the dideoxy chain-termination method using T₅ and T₇ primers (Stratagene)
and the fmol DNA sequencing system (Promega). DNA sequences were analyzed using Genetics Computer Group (Madison, WI) software (Devereux et al., 1984).

**Expression of Coccomyxa CA in E. coli and Protein Isolation**

A 786-bp fragment containing the complete coding region for the Coccomyxa CA, including an extra 86-bp 3' untranslated region, was obtained by PCR amplification using the primers 5'-GCCGAATTTCAAGGCAGCCCATGGTGAAGCAGCTAAAGACACTGCC-3' and 5'-CTCCATCTAGAGTCACCTTGGAGGCA-3', which contain cleavage sites for EcoRI and XbaI, respectively. The PCR product was digested with EcoRI and XbaI, and then ligated into the expression vector pMAL-c2 (New England Biolabs) just downstream of and in-frame with the maltE gene encoding the MBP. The resulting MBP/β-CA was expressed in E. coli and purified using an amylose resin (New England Biolabs). For enzyme production, the cells were grown in Luria-Bertani broth containing 0.2% Glc and 50 mM EDTA.

Samples of 20 mg cells were grown in and purified using an amylose resin (New England Biolabs) to verify that the N terminus was correct after cleavage of the fusion protein. The purified CA was sequenced using the primers 5'-AGAGTCACCTTGTAGGCA-3' and 5'-CTCCATCTAGAGTCACCTTGGAGGCA-3', which contain cleavage sites for EcoRI and XbaI, respectively. The PCR product was digested with EcoRI and XbaI, and then ligated into the expression vector pMAL-c2 (New England Biolabs) just downstream of and in-frame with the maltE gene encoding the MBP. The resulting MBP/β-CA was expressed in E. coli and purified using an amylose resin (New England Biolabs). For enzyme production, the cells were grown in Luria-Bertani broth containing 0.2% Glc and 50 mM EDTA.

**Protein Isolation**

The cells were harvested by centrifugation at 4,000 g at 4°C, and resuspended in 50 mL of 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA (column buffer) before being disrupted in a precooled French pressure cell. Intact cells and cell debris were removed by centrifugation at 14,000g. The supernatant was diluted five times in column equilibration buffer and loaded on an amylose-resin column preequilibrated with column buffer. The sample was concentrated using a Centriprep 30 unit (Amicon, Beverly, MA) and incubated with column buffer at a flow rate of 1 mL min⁻¹. After washing with eight column-volumes of column buffer, the fusion protein was eluted with 10 mM maltose in the same buffer. The sample was concentrated using a Centriprep 30 unit (Amicon, Beverly, MA) and incubated for 10 h with factor Xa (Boehringer Mannheim) at a final concentration of 0.5%. The cleaved fusion protein was desalted using a PD-10 column (Pharmacia) before being loaded on a Q-Sepharose FF (Pharmacia) ion-exchange column equilibrated with 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Coccomyxa CA was eluted with a stepwise gradient of 20 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, and 1 mM EDTA. Fractions containing CA activity were pooled and loaded for one more passage through the affinity column. The isolated protein was concentrated using a Centricron 10 device (Amicon), and fractions containing CA activity throughout the purification steps were analyzed by SDS-PAGE (Laemmli, 1970). The purified CA was sequenced using a sequencing system (model 476A, Applied Biosystems) to verify that the N terminus was correct after cleavage of the fusion protein.

**Preparation of Antiserum**

The purified Coccomyxa CA, in PBS (10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 2.5 mM KCl), was mixed with Freund’s complete adjuvant and injected into rabbits (Agrisera AB, Vännäs, Sweden). Every 14 d the immunized rabbits were injected with another 100 μg of CA mixed with incomplete adjuvant.

**Protein Concentration**

Protein concentrations were determined either according to the method of Bradford (1976) or by determining the A₄₅₀ for the purified enzyme. The molar extinction coefficient (ε) for Coccomyxa CA was determined as described by Gill and von Hippel (1989), giving a value of ε₂₈₀ = 37,500 ± 3,500 M⁻¹ cm⁻¹. All stated enzyme concentrations are subunit concentrations.

**Protein Structure Analyses**

The native molecular mass of the purified Coccomyxa CA was estimated by gel-filtration chromatography performed on a Sephacryl S-300H column (Pharmacia) equilibrated with 20 mM Tris-HCl and 0.1 mM NaCl, pH 7.4. Ovalbumin (45 kD), aldolase (158 kD), catalase (240 kD), and ferritin (450 kD) (Combithek, Boehringer Mannheim) were used as protein standards. Purified Coccomyxa CA and soluble cell proteins were analyzed by 9% polyacrylamide native-PAGE. The proteins were blotted onto a nitrocellulose filter for immunoreaction tests with antiserum directed against CA from Coccomyxa in conjunction with horseradish peroxidase-conjugated secondary antibodies and an enhanced-chemiluminescence detection system (Amersham).

CD spectra for Coccomyxa CA were measured on a spectropolarimeter (model J-7720, Jasco, Easton, MD) at 23°C. Each spectrum shown was the result of three scans using a bandwidth of 1 nm. For far-UV-region analysis the protein concentration was 0.25 mg mL⁻¹ and the path length was 1 mm. For near-UV analysis the protein concentration was 1.0 mg mL⁻¹ and the path length was 4 mm. Spectra recorded for pea CA were obtained using the same protocol except that the far-UV region was scanned in a spectrodichrograph (model CD6, Jobin-Yvon Instruments SA, Longjumeau, France) using a sample concentration of 0.5 mg mL⁻¹ and a 0.5-mm path length. The samples contained 10 mM potassium phosphate buffer, pH 7.5. The observed ellipticities were converted to mean residue ellipticities (θ) on the basis of a molecular mass of 24.7 kD and 227 amino acids for Coccomyxa CA and soluble cell proteins. CA Activity Measurements

During enzyme purification, fractionation, activation, and inhibition studies, CO₂-hydration activity was assayed at 2°C using the colorimetric method of Rickli et al. (1964). Initial rates of CO₂ hydration were measured at 578 nm using a sequential stopped-flow spectrophotometer (model DX-17MV, Applied Photophysics, Leatherhead, UK) at 25°C by the changing-pH method ( Khalilah, 1971; Steiner et al., 1975). The buffer/indicator pair was Taps (3-[2-hydroxy-1,1-bis[(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid)/m-cresol purple with 10 μM EDTA. The initial rates, calculated by fitting data from the first part of the trace to a first-order rate equation, were fitted by
Subcellular Fractionation

A 10% to 80% linear Percoll gradient was generated by mixing 100% Percoll with 2× breaking medium (1× breaking medium: 35 mM Hepes-KOH, pH 7.7, 375 mM sucrose, 10 mM EDTA, 1 mM MnCl₂, and 5 mM MgCl₂) in a 1:1 ratio and centrifuging the solution at 40,000 g for 1 h. One liter of Coccomyxa culture (5 µg mL⁻¹ chlorophyll) was centrifugated at 1,500 g for 10 min at 4°C and resuspended in 20 mL of 1× breaking medium. Cells were disrupted for 30 s in a precooled Bead Beaker (Biospec Products, Bartlesville, OK) filled with 0.5-mm-diameter glass beads and cell suspension (1:1 [v/v]). The disrupted cells were carefully layered onto the Percoll gradient and centrifuged at 3,000 g for 20 min at 4°C. Four distinct fractions were taken, each characterized with a light microscope.

Marker enzyme activities were measured at 25°C in all subcellular fractions. The chloroplast stromal marker NADP-GAPDH was measured according to the method of Winter et al. (1982), except that 4 mM DTT rather than GSH was included in the assay medium to ensure full activation of the enzyme. PEPC was measured as a marker for the cytosol (Gardestrom and Edwards, 1983). For all marker-enzyme/activity measurements, changes in \( A_{412} \) caused by formation of a 2-nitro-5-thiobenzoate anion were followed by extensive dialysis against degassed phosphate buffer under \( N_2 \).

RESULTS

Cloning and Sequencing of β-CA cDNA

Semipurified intracellular CA isolated from Coccomyxa cells as previously described (Hiltonen et al., 1995) was digested with trypsin and the resulting peptides were separated by HPLC. Five different internal amino acid sequences were determined (Fig. 1). One of the sequences, TAGVTNLW, was used to design a degenerated 18-base primer. Along with a 22-base primer specific for the T₇ promoter, the degenerate oligonucleotide was used to amplify a 550-bp fragment from the Coccomyxa CDNA library. Subsequent sequencing of the fragment confirmed that it was derived from a CDNA encoding part of a β-CA gene.

The 550-bp fragment was used as a specific β-CA probe to screen the Coccomyxa CDNA library, from which 15 positive clones were obtained. Three of the longest cDNAs were completely sequenced in both directions, and all three were identical except for different amounts of truncation at the 5’ end. The longest of the three selected clones was 1137 bp, consisting of an open-reading frame of 681 bp, with 198 and 258 untranslated nucleotides in the noncoding 5’ and 3’ regions, respectively. The CDNA library was rescreened with a probe corresponding to the 5’ end of the CA CDNA. This fragment starts at amino acid position 23 and stops 570 bp downstream. The second screening gave an addi-
Overexpression of Coccomyxa CA in E. coli homologs.

about 50% similar (26%–30% identical) to higher-plant and Coccomyxa and two slightly weaker negative bands at 208 and 218 nm. The enzyme has an intense positive band at 194 nm Coccomyxa buffer and pH dependencies. Levels of inhibition of the paratively small and could reflect subtle differences in the higher-plant CA. The differences are comparatively small and could correspond to a similar-sized band in the crude extract of (8.0 \text{ s}^{-1}) was determined using the stopped-flow technique. Values of (V_{e} - V_{o})/(V_{t} - V_{o}) where \( V_{e} \) is the elution volume, \( V_{o} \) is the column void volume, and \( V_{t} \) is the total bed volume.

**Figure 2.** Purification and native molecular mass estimation of Coccomyxa CA expressed in E. coli. A. Samples from the purification steps were analyzed by SDS-PAGE. The gel was stained with Coomassie blue G-250. Lane 1, Uninduced cell extract (10 \( \mu \)g); lane 2, induced cell extract (10 \( \mu \)g); lane 3, first amylose resin chromatography (10 \( \mu \)g); lane 4, factor Xa digestion (5 \( \mu \)g); lane 5, Q-Sepharose chromatography (2 \( \mu \)g); and lane 6, second amylose resin chromatography (2 \( \mu \)g). B. Purified Coccomyxa CA analyzed by size-exclusion chromatography using a Sephacryl S-300H column (Pharmacia). \( K_{d} = (\text{Ve} - \text{Vo})/(\text{Vt} - \text{Vo}) \) where \( \text{Ve} \) is the elution volume, \( \text{Vo} \) is the column void volume, and \( \text{Vt} \) is the total bed volume.

**CO2-hydration activity of Coccomyxa CA caused by specific inhibitors are presented in Table 1, together with \( K_{i} \) values for the pea homolog. A relatively large difference was observed between the algal and pea enzymes in their sensitivity to the sulfonamide inhibitors. The binding affinity of ethoxyzolamide is almost 30 times higher for the pea enzyme than for the Coccomyxa CA, whereas the binding affinity of acetazolamide is more than 10 times higher for the algal protein. The inhibition by anions showed only minor variations between the two CAs.

**Oxidation/Reduction of Coccomyxa CA**

\( \beta \)-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). Oxidized pea CA.

Structural Analysis of Coccomyxa CA

In the far-UV region (Fig. 3A) the CD spectrum of the Coccomyxa enzyme has an intense positive band at 194 nm and two slightly weaker negative bands at 208 and 218 nm. This band pattern suggests that Coccomyxa CA has a high \( \alpha \)-helix structure content (Johnson, 1990). The higher-plant homolog from pea also seems to be dominated by \( \alpha \)-helix structures, and the spectra for the two enzymes suggest a similar content of various secondary structure elements (Fig. 3A). In the near-UV region there are extensive differences: Coccomyxa CA has a predominately positive CD spectrum, whereas the spectrum for pea CA is dominated by a negative band at around 280 nm (Fig. 3B).

**Kinetic Properties**

The Coccomyxa CA was found to have a high catalytic activity. Kinetic parameters for the CO2-hydration reaction were determined using the stopped-flow technique. Values of \( K_{cat} = (3.8 \pm 0.1) \times 10^{7} \text{ s}^{-1} \) and \( K_{m} = 4.7 \pm 0.3 \text{ mm} \) were obtained in 50 mm Taps buffer, pH 8.7, at 25°C. These are somewhat higher than the values reported for the pea CA (Johansson and Forsman, 1993), whereas the \( K_{cat} = K_{m} \) ratio of (8.0 \( \pm \) 0.5) \( \times 10^{7} \) \( \text{m}^{-1} \text{s}^{-1} \) is almost identical to that reported for the higher-plant CA. The differences are comparatively small and could reflect subtle differences in buffer and pH dependencies. Levels of inhibition of the

1. Overexpression of Coccomyxa CA in E. coli
2. Structural Analysis of Coccomyxa CA
3. Kinetic Properties
4. Oxidation/Reduction of Coccomyxa CA
required a reducing agent for maximal activation of the enzyme (Johansson and Forsman, 1993). In contrast, the activity of *Coccomyxa* CA was found to be independent of the presence of a reducing agent. The CO₂-hydration activity of enzyme purified without reductant in the isolation buffers was high, and it remained constant when the enzyme was incubated for 10 min in 100 mM 2-mercaptoethanol or 1 mM DTT (data not shown). Thus, the catalytic activity of *Coccomyxa* CA is not significantly affected by the oxidation state. Analysis of the amino acid sequence for *Coccomyxa* CA (Fig. 1) shows the presence of nine Cys residues in each subunit. Of these, only the two Cys residues thought to act as zinc ligands are conserved.

The accessibility of the Cys residues was determined by the addition of DTNB to oxidized and reduced enzyme. The overexpressed enzyme purified from *E. coli* was assumed to be oxidized because of the absence of reductants in the isolation buffers. The CO₂-hydration activity of enzyme purified without reductant in the isolation buffers was high, and it remained constant when the enzyme was incubated for 10 min in 100 mM 2-mercaptoethanol or 1 mM DTT (data not shown). Thus, the catalytic activity of *Coccomyxa* CA is not significantly affected by the oxidation state. Analysis of the amino acid sequence for *Coccomyxa* CA (Fig. 1) shows the presence of nine Cys residues in each subunit. Of these, only the two Cys residues thought to act as zinc ligands are conserved.

The accessibility of the Cys residues was determined by the addition of DTNB to oxidized and reduced enzyme. The overexpressed enzyme purified from *E. coli* was assumed to be oxidized because of the absence of reductants in the isolation buffers. The enzyme was reduced by incubation with 10 mM DTT or 100 mM 2-mercaptoethanol for 1 h, followed by dialysis under a cushion of N₂ to remove excess reducing agent. Reacting the oxidized *Coccomyxa* CA with DTNB gave a molar ratio of modified Cys residues per subunit of 3.9 ± 0.1. Assuming that two Cys residues are zinc ligands, and thus are inaccessible to DTNB, it follows that three Cys residues within the oxidized enzyme did not react with DTNB. In the reduced enzyme, the molar ratio of modified Cys residues per subunit was 5.6 ± 0.3, indicating that one to two extra Cys residues are accessible after reduction, although at least one residue remains inaccessible in the reduced state. These results suggest that the oxidized *Coccomyxa* CA contains a disulfide bridge that can be broken upon reduction. Moreover, the formation of this disulfide does not affect the enzymatic activity. The mobility of *Coccomyxa* CA in gel electrophoresis under denaturing conditions is not affected by the presence or absence of reducing agents in the sample buffer, indicating that no disulfide bridges are formed between subunits (data not shown).

Cell-Fractionation Studies

Microscopic studies of the fractions generated by separation in a Percoll gradient showed an enrichment of seemingly intact chloroplasts in fraction 3, although it also contained aggregated material, probably derived from thylakoid membranes. Fraction 1 contained soluble proteins, derived from both the cytosol and broken organelles; fraction 2 contained thylakoid membranes; and fraction 4, the lowest fraction, contained intact cells. Measurements of marker-enzyme activities supported the microscopic observations (Table II). Most of the activity of both NADP-GAPDH (95%) and PEPC (100%) were detected in fraction 1, whereas no enzyme activity was detected in fractions 2 and 4. Fraction 3 contained activity for the chloroplast marker enzyme NADP-GAPDH (5%) but no PEPC activity. This confirms the presence of intact chloroplasts in fraction 3. Of the four fractions, CA activity was found only in fraction 1. Western-blot analysis confirmed the CA activity measurements, with CA protein detected only in fraction 1 (Fig. 4). No protein corresponding to *Coccomyxa* CA was detected in fraction 3, the fraction containing intact chloroplasts.

DISCUSSION

We have isolated and characterized a cDNA encoding a β-CA from the alga *Coccomyxa*. Enzymes from the β-CA family share some common features with members of the other two CA families, α-CAs and γ-CAs. They are all zinc enzymes that catalyze the reversible dehydration of HCO₃⁻ to CO₂, and they are all sensitive to similar kinds of inhibitors. We assayed the CO₂-hydration activity of recombinant *Coccomyxa* CA by colorimetry according to the method of Rickli et al. (1964) with a pH change from 8.2 to 6.5. The reactions were followed at 2°C in 10 mM barbital buffer in the presence of inhibitor at different concentrations. Data for pea CA are from Johansson and Forsman (1993).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kᵢ (μM)</th>
<th>Kᵢ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyzolamide</td>
<td>11 ± 1</td>
<td>0.4</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>2.1 ± 0.5</td>
<td>28</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>6.6 ± 0.7</td>
<td>20</td>
</tr>
<tr>
<td>N₃⁻</td>
<td>17 ± 3</td>
<td>6</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>48,000 ± 5,000</td>
<td>40,000</td>
</tr>
</tbody>
</table>

Table I. Inhibition of *Coccomyxa* CA by sulfonamides and anions

CO₂-hydration activity was assayed by colorimetry according to the method of Rickli et al. (1964) with a pH change from 8.2 to 6.5. The reactions were followed at 2°C in 10 mM barbital buffer in the presence of inhibitor at different concentrations. Data for pea CA are from Johansson and Forsman (1993).
chemical inhibitors, including sulfonamides and monovalent anions. Nevertheless, the α−, β−, and γ-CAs clearly belong to three distinct gene families according to sequence homology (Hewett-Emnett and Tashian, 1996). On the same basis, β-CAs can be further divided into three subgroups: those originating from eubacteria, dicot plants, and monocot plants (Hewett-Emnett and Tashian, 1996). Alignment of the deduced amino acid sequence for the Coccomyxa CA with other known β-CAs, however, shows that this enzyme cannot readily be classed with any of these subgroups.

According to both subcellular fractionation and western-blot analysis, it appears that the subcellular localization of Coccomyxa CA is extrachloroplastic. The marked decrease in the PEPC:NADP-GAPDH ratio indicates little or no contamination of cytoplasm in the chloroplast fraction. If CA were chloroplastic, the CA:NADP-GAPDH ratio would increase in the chloroplast fraction compared with the soluble fraction (Table II). Instead, a distinct decrease was observed, clearly indicating an extrachloroplastic localization of CA. This is supported by western-blot analysis, in which no CA protein could be detected in the chloroplast fraction (Fig. 4, lane 3). Because of the lack of an obvious transit peptide, there is no indication at present of any other localization for the Coccomyxa CA than in the cytosol. Furthermore, the isolated cDNA is almost certainly full length, since the 200-bp 5′-untranslated region upstream from the putative start Met does not contain any additional Met codons before the stop codons in any of the three frames. The 5′ ends of 12 positive clones were also analyzed and all sequences were found to be identical except for different amounts of truncation, which strongly indicates that the sequence was reliably identified. In summary, the majority of CA activity in Coccomyxa is located in the cytosol, although the presence of as-yet-unidentified chloroplastic or mitochondrial CAs cannot be excluded.

The specific function of a cytosolic CA in Coccomyxa is unclear at this time. In a previous study Palmqvist et al. (1995) suggested that this CA was chloroplast located and that it had a role similar to that of CA in C₃ plants. A cytosolic CA could also facilitate the diffusion of inorganic carbon from the inner surface of the plasmalemma to the chloroplast envelope (Badger and Price, 1994). Moreover, the absence of a CCM in Coccomyxa has previously been correlated with the relatively more efficient Rubisco of this alga than that of algae possessing a CCM (Palmqvist et al., 1995). Palmqvist et al. (1995) also suggested that there was an extracellular CA, but so far we have been unable to measure any periplasmic CA activity from intact Coccomyxa cells. Another possibility is that the CA in Coccomyxa may not be directly involved in photosynthesis. As suggested by Fett and Coleman (1994), cytosolic CA may instead be required to catalyze the formation of HCO₃⁻, the substrate for cytosolic PEPC, in a role similar to that suggested for the CA localized in the mesophyll cells of C₄ plants.

The tricarboxylic acid cycle is the source of carbon skeletons for many growth processes. If the pool of intermediates in the cycle undergoes a net loss, oxaloacetate will not be regenerated. However, the mechanism whereby oxaloacetate is formed by carboxylation of PEP allows the tricarboxylic acid cycle to be replenished for continued operation. A cytosolic CA was recently identified in potato leaves (Rumeau et al., 1996), suggesting that the existence of cytosolic β-CA may be common to both algae and higher plants.

The CA from Coccomyxa is at least as efficient a catalyst as higher-plant CAs such as those from pea (Johansson and Forsman, 1993) and spinach (Rowlett et al., 1994). Similarly, we observed structural features common to the different β-CAs. The primary structures contain a high degree of identity. The content of secondary structure elements seems to be similar, because the general outline of the CD spectra in the far-UV region is very similar, suggesting a domination of α-helix structure. This highlights one of the structural differences between the α- and γ-CAs: the α- and γ-CAs are composed mainly of β-sheet structures (Kannan et al., 1975; Eriksson et al., 1988a; Eriksson and Liljas, 1993; Boriack-Sjodin et al., 1995; Kisker et al., 1996). However, Coccomyxa CA also shows several distinct props.

### Table II. Distribution of marker-enzyme activities in a soluble fraction (fraction I) and an enriched chloroplast fraction (fraction 3)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NADP-GAPDH</th>
<th>PEPC</th>
<th>CA</th>
<th>PEPC:NADP-GAPDH</th>
<th>CA:NADP-GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>43.9</td>
<td>5.8</td>
<td>45.1</td>
<td>0.13</td>
<td>1.03</td>
</tr>
<tr>
<td>Chloroplast</td>
<td>2.6</td>
<td>&lt;0.02</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

*a* 1 unit= 1 activity unit as described by Rickli et al. (1964). *b* Activities of PEPC and CA were below the detection limit.

**Figure 4.** Western-blot subcellular fractions separated by SDS-PAGE and analyzed by immunoblotting using antisera specific for Coccomyxa CA. Lane 1, Soluble fraction (5 μg of protein); lane 2, thylakoid fraction (5 μg); and lane 3, chloroplast fraction (5 μg).
roperties that imply certain differences between the algal and higher-plant β-CAs.

There are differences in quaternary structure; the Coccomyxa CA is a homotetramer, whereas CAs from C₃ dicots apparently are homoooligomers (Aliev et al., 1986; Björckbacka et al., 1997). Furthermore, the near-UV CD spectra of CAs from Coccomyxa and pea differ extensively; the CD bands in this wavelength region arise mainly from immobilized aromatic side chains located in an asymmetric environment (Strickland, 1974). This region is generally assumed to be indicative of the tertiary structure of the protein. However, because the Coccomyxa and pea enzymes differ in Trp content (having five and two Trp residues, respectively), and the enzymes apparently possess distinct quaternary structures, the different shapes of the near-UV CD spectra do not necessarily imply different overall folding of the individual subunits. Furthermore, we have studied pea CA mutants that assemble into tetramers rather than wild-type octamers, and these mutants have predominantly positive CD spectra in the near-UV region, with shapes and intensities very similar to those of Coccomyxa CA (Björckbacka et al., 1997).

The enzymatic activity of the Coccomyxa CA was found to be independent of a reducing environment. CAs from the two prokaryotes E. coli and Synechococcus sp. PCC 7942 are similarly insensitive to oxidation (Guilhoton et al., 1992; Price et al., 1992), whereas the CAs in pea and other C₃ dicots are dependent on a reducing environment to retain catalytic activity. Of the nine Cys residues in the Coccomyxa CA, only the two proposed zinc ligands are conserved. Under oxidizing conditions, the Coccomyxa CA apparently forms a single disulfide bond. Only four Cys residues were modified by DTNB in the oxidized enzyme, whereas five to six Cys residues were modified in the reduced Coccomyxa CA. This bond is probably not formed within the active-site region. Therefore, the catalytic activity of the Coccomyxa CA remains unchanged whether the enzyme is oxidized or reduced.

The Coccomyxa CA activity is inhibited by generally recognized CA inhibitors. However, the relative sensitivities of the Coccomyxa and pea CAs to the two sulfonamides used in this study differ. The more hydrophilic sulfonamide acetazolamide binds more strongly to the Coccomyxa protein, whereas the more hydrophobic ethoxzolamide has a stronger affinity for the pea CA. Because both CAs are equally efficient catalysts, it is unlikely that there are any significant structural differences involving the catalytically active residues. In the human CA II, the sulfonamide nitrogen ion has been shown by crystallographic studies to bind to the zinc ion by replacing the hydroxide ion, resulting in the aromatic or heterocyclic part of the sulfonamide being oriented toward the hydrophobic side of the active site (Eriksson et al., 1988b). Assuming that the sulfonamide coordination to the zinc is similar in the β-CAs, the weaker binding of the more hydrophobic (and the stronger binding of the more hydrophilic) sulfonamide to the Coccomyxa CA than to the pea enzyme may reflect differences in the hydrophobicity of the surfaces near the active site. This could be limited to differences in one or a few residues interacting with the aromatic part of the inhibitor.

At present the only β-CAs that have been catalytically investigated are from the higher plants pea (Johansson and Forsman, 1993) and spinach (Pocker and Ng, 1973; Rowlett et al., 1994). In general, the enzymatic characteristics for these β-CAs are consistent with the zinc-hydroxide mechanism proposed for α-CAs (Steiner et al., 1975), and it seems likely that the Coccomyxa CA also follows the same general mechanism. The work presented here will provide a foundation for a more detailed characterization of the physiological function of CA in Coccomyxa under different growth conditions, especially in comparison with the CA homologs in algae possessing a CCM.

ACKNOWLEDGMENT

We thank Dr. Bo Ek (Department of Cell Research, Swedish University of Agricultural Sciences, Uppsala, Sweden) for skillful determination of the amino acid sequences.

Received February 12, 1998; accepted May 5, 1998.
Copyright Clearance Center: 0032–0889/98/117/1341/09.

LITERATURE CITED

Intracellular Carbonic Anhydrase of Coccomyxa

Eriksson AE, Kylsten PM, Jones TA, Liljas A (1988b) Crystallographic studies of inhibitor binding sites in human carbonic anhydrase II: a pentacoordinated binding of the SCN⁻ ion to the zinc at high pH. Proteins Struct Funct Genet 4: 283–293


