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Structural Characterization of Beta Carbonic Anhydrases From Higher Plants.

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STRUCTURAL CHARACTERIZATION OF BETA CARBONIC ANHYDRASES FROM HIGHER PLANTS

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

Michael H. Bracey
B.A., Louisiana State University, 1991
May, 1998
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ABSTRACT

It is the goal of this dissertation research to reveal some aspects of the physical nature of spinach carbonic anhydrase as a representative βCA using the techniques of sequence comparison, molecular biology, and biophysics. Though both α and β carbonic anhydrases are zinc dependent metalloenzymes, it is clear that the two isoforms do not adopt the same mechanism for coordinating the active site metal. While αCA binds zinc through three histidine ligands, βCA cannot due to a lack of evolutionarily conserved histidines. Instead, the β family has adopted a ligand scheme incorporating a single histidine and two cysteines. This has been determined by systematically mutating possible zinc ligands in the spinach enzyme and then assaying the resulting variants for stoichiometric metal binding. Additionally, this conclusion is corroborated by inspection of the wild type enzyme's extended X-ray spectrum. This analysis indicates the metal is surrounded by two sulfur atoms and two nitrogen or oxygen species.

Secondly, it has been long established that not only do the β isoforms differ from their α cousins in their multimeric assembly, but subtypes exist within the β family in which monocot forms assemble into lower molecular weight oligomers while dicot forms assemble into higher order structures. In an attempt to gain insight into the differences between monocot and dicot CAs, the CA cDNA from barley, a moncot, was sequenced. Analysis of the open reading frame revealed that the barley enzyme lacked ten amino acids at the carboxyl terminus which are conserved in the dicot isozymes. It is here demonstrated that this extension contributes to the difference in multimeric organization between monocots and dicots. When this extension is deleted from the spinach enzyme,
the resulting mutant displays an apparent deficit in its ability to form higher order multimers. Furthermore, this carboxyl extension will interact with the CA holoenzyme in the yeast two-hybrid system showing that the observed characteristics of the deletion mutant do not arise from secondary disruptions, but rather the carboxyl terminus does participate in intermolecular interactions.
CHAPTER 1
LITERATURE REVIEW

The carbonic anhydrases (CA) represent a diverse and widespread family of enzymes found in all phylogenetic branches of life so far examined, except for the fungi (Lindskog, 1997). Presently, this family is divided into three subgroups based on the primary structure of the proteins, and these subgroups are designated as the α, β, and γ families (Hewett-Emmett & Tashian, 1996). Though these isoforms do not share any appreciable sequence homology with one another, they do share some striking common features. All CAs are zinc dependent metalloenzymes which catalyze the hydration of carbon dioxide to form bicarbonate and a proton. In this reaction, a zinc bound water molecule dissociates to generate a zinc bound hydroxyl and a free proton. This proton is shuttled to the solvent and the remaining OH⁻ attacks CO₂ to form bicarbonate. Once this product leaves the active site and a new water molecule binds to the zinc, the reaction starts over again. Thus, the role of zinc in the reaction scheme is to generate reactive OH⁻. (Banci et al., 1990). Because the reaction converts gaseous CO₂ to an ion and generates H⁻, CAs play a role in many systems to regulate pH and trap inorganic carbon in the aqueous phase (Lindskog, 1997). This enzyme family also displays extraordinarily fast reaction kinetics, with k_cat values of 10⁶ sec⁻¹ for isozymes such as human CA II (Behravan et al., 1990).

The α Carbonic Anhydrases

The αCAs are by far the most studied and best characterized of the CA families. These enzymes were formerly known as the mammalian or eukaryotic isozymes, as they
are found in various tissues in all mammals examined. Among vertebrates, seven isozymes have been identified, and they are designated as CA I through CA VII. They differ from one another in both their reaction kinetics and tissue distributions. Some members of this family are membrane bound, but most αCAs are monomeric, soluble proteins. Among these isozymes, the CAs are crudely divided into either the "high" or "low" activity forms based on their kinetic profiles. Additionally, αCAs are seemingly unique among the three families in that they display esterase activity in addition to their CO₂ hydration catalysis (Holmes, 1977).

CA II is the most thoroughly studied of the αCAs. It is widely distributed in mammalian tissues and can be found in the cytoplasm of epithelial tissues, red blood cells, brain, eye, pancreas, kidney, liver, stomach, bone, and uterus (Tashian, 1989). Among its ascribed physiological roles, CA II is believed to mediate the exchange of CO₂ in both the lungs and in capillary beds, buffer the plasma, acidify bodily fluids (such as gastric juices and urine), and aid the reabsorption of bicarbonate in the kidney. CA II is classified as a high activity isozyme and has been compared to the low activity CA I in attempts to elucidate the structural features which distinguish the two kinetic forms (Behravan et al., 1990; Behravan et al., 1991).

CA I and CA III are both classified as low activity isozymes, and they are found primarily in cytosols of red cells and skeletal muscle, respectively. They display turnover numbers between one fifth and one hundredth that of CA II (Behravan et al., 1991; Tu et al., 1990). These enzymes, like CA II, were thought to play important roles in the exchange of CO₂ from the blood to either tissues or the alveoli airspaces (Tashian, 1989). Contradictory to this notion is the observation that a human deficiency in CA I
does not lead to any observable clinical pathology (Kendall & Tashian, 1977). It would therefore seem that CA I serves some sort of backup role for CA II despite the fact that it is the major non-hemoglobin protein in erythrocytes (Lindskog, 1997).

The remaining CA isozymes are less well characterized. CA VII is a soluble cytoplasmic enzyme. It has been found in salivary glands and was originally reported to be membrane bound (Tashian, 1989). It is presently believed to play a role in the secretion of bicarbonate into the saliva. A recombinant form of this enzyme has been overexpressed, and it displays less than half of the activity of CA II (Lakkis et al., 1996). CA VI is also synthesized in salivary glands but with a signal sequence which directs its export from the cell. The enzyme itself is present in oral secretions where it is believed to play a role in the maintenance of pH (Lindskog, 1997).

CA IV is also secreted but remains membrane bound in the lung and kidney via a phosphatidylinositol anchor (Zhu & Sly, 1990). It is reported to contain at least one disulfide linkage necessary for enzymatic activity, and is devoid of O-linked sugars. The bovine form, however, purportedly bears five to six N-linked sugars in contrast to its unadorned human counterpart (Zhu & Sly, 1990). These authors also cite possible roles for CA IV including facilitating CO₂ efflux from alveoli and aiding bicarbonate reabsorption in proximal tubules.

Lastly, CA V is a soluble isozyme found in the matrix of the mitochondria. This localization is conferred by an amino terminal extension which directs its post-translational transport into the organelle. CA V is presumed to provide bicarbonate as a substrate for gluconeogenesis (Dodgson & Forster, 1986).
In addition to the vertebrate isozymes, αCAs have been described in unicellular species as well. Most notable among these is the photosynthetic alga *Chlamydomonas reinhardtii*, which contains at least three α isozymes (Lindskog, 1997). Two of these, designated CAH1 and CAH2, are located in the periplasmic space and are believed to facilitate the concentration of CO$_2$ for photosynthetic carbon fixation (Fukuzawa et al., 1990; Rawat & Moroney, 1991). In fact, CAH1 gene expression is rapidly upregulated when cultures are shifted to low CO$_2$ tensions, and this response has been shown to be part of a complex CO$_2$ concentrating mechanism designed to minimize photorespiration (Fukuzawa et al., 1990). Curiously, the *Chlamydomonas* enzymes are post-translationally processed into a large and a small subunit which remain associated via disulfides in the tetrameric holoenzyme (Kamo et al., 1990).

Based on a variety of X-ray crystal structures and the extraordinary amino acid identity among the vertebrate CAs, it is believed that all α isozymes possess nearly identical tertiary structures. Inspection of representative structures, such as that for human CA II, reveal many features of this enzyme family (Figure 1.1). The protein is roughly spherical and rich in beta strands. Most of these strands form a single twisting sheet which dominates the enzyme’s overall structure. It is this sheet that forms the wall of a funnel on the surface of the enzyme which leads down to the active site approximately 15 Å below the mean surface (Lindskog, 1997).

At the bottom of this funnel sits the active site zinc atom coordinated to three histidine residues. It is this environment which accounts for CA’s extraordinary kinetics compared to zinc metal in an exposed aqueous environment (Banci et al., 1990; Håkansson et al., 1992). One side of the cavity is hydrophilic and therefore attracts both
Figure 1.1 Structure of human CA II. This structure was rendered with RasMol by Roger Sayle using the coordinates 2cba deposited in the Protein Data Bank by Håkansson et al. (1992).
water and bicarbonate to facilitate their passage to and from the active site. Additionally, on this wall sit the residues thought to play important roles in shuttling a proton from the active site to the outside environment during the generation of active site hydroxide from a zinc bound water (Banci et al., 1990). Conversely, the opposite wall of the funnel is rich in hydrophobic residues. This side is believed binding of carbon dioxide and positioning the molecule for attack during hydration (Håkansson et al., 1992).

Along the funnel leading to the αCA active site are a number of residues which are invariant, but still some are found to correlate with specific isozymes. In an effort to understand these differences, various papers have appeared in which “wall mounted” residues have been selectively targeted for mutagenesis in an attempt to re-engineer one CA isozyme into another. These efforts have revealed the importance of such residues as threonine 200 in CA II and how it relates to this isozyme’s remarkable kinetics, while at the same time demonstrating that apparent distinctions such as a conspicuous asparagine to valine substitution at position 62 have no readily apparent consequences (Behravan et al., 1990; Behravan et al., 1991).

The β Carbonic Anhydrases

The β family of carbonic anhydrases is less well characterized than the α isozymes. From a structural point of view, this is largely due to the lack of an X-ray crystal structure for this class. Formerly, this subtype was identified as the prokaryotic or bacterial family, as representatives are found in both unicellular prokaryotes and the chloroplasts of higher plants. Based on sequence analysis and oligomeric assembly, the β forms are subdivided into three subtypes: the dicot, monocot, and bacterial isozymes. All
three of these share extensive sequence homology, but subtle differences can be
discerned (Hewett-Emmett & Tashian, 1996).

The bacterial class of βCAs is represented in *E. coli* and *Synechococcus*. The*
cynT* gene product in *E. coli* displays CO$_2$ hydration activity and is induced by growth in
the presence of cyanate. As a product of a cyanate-inducible operon, *cynT* is thought to
to mediate the ensure a ready supply of bicarbonate to be used as a substrate by cyanase
in the detoxification of cyanate to ammonia and carbon dioxide (Guilloton et al., 1992).
The hydration of CO$_2$ by *cynT* would thus stimulate the forward reaction of cyanase by
both supplying reactants and removing products simultaneously. Unlike the αCAs, *cynT*
exists as an oligomer, and the multimeric state of the enzyme is reportedly influenced by
the availability of bicarbonate (Guilloton et al., 1992). During gel filtration
chromatography, the enzyme was found to shift to a lower molecular weight when the
column was developed in the presence of bicarbonate.

The *icfA* gene product in *Synechococcus* PCC7942 is also a βCA. This gene was
identified in a screen for mutants which require elevated CO$_2$ concentrations to maintain
carbon fixation (Fukuzawa et al., 1992). Apparently, *icfA*, like the periplasmic CAH1 of
*Chlamydomonas*, ensures a ready supply of CO$_2$ for photosynthesis by this autotroph.

The remaining βCAs are divided between the monocots and the dicots. Among
the latter group, cDNAs encoding isozymes have been cloned from pea, spinach,
tobacco, aspen, *Arabidopsis*, and several species of *Flaveria* (Hewett-Emmett &
Tashian, 1996; Larsson et al., 1997). Additionally, evidence for CA protein has been
provided from parsley (Tobin, 1970). These isozymes generally display reaction kinetics
comparable to their α counterparts. For example, the spinach enzyme has a $k_{cat}$ of 2 x
10^3 \text{sec}^{-1}$, equivalent to human CA I (Rowlett et al., 1994). However, βCAs do display altered susceptibility to αCA inhibitors such as the sulfonamides and heavy metals. For example, spinach CA exhibits a $K_i$ for acetazolamide that is more than $10^3$ times weaker than αCAII (Pocker & Ng, 1974). This difference in inhibition may reflect a difference in the active site architectures of members of these two families.

The dicot isozymes are distinguished by their high native molecular weight. The exact oligomeric makeup of these holoenzymes has long been a point of contention. Though they are clearly made up of identical subunits, different techniques have yielded various native molecular weights, confounding attempts to identify how many subunits make up the enzyme (Graham et al., 1984). Recently, studies on the pea enzyme using bifunctional cross-linking agents strengthen the case for an octameric complex (Björkbacka et al., 1997). It may be the case that βCA assumes a non-ideal shape, as would be the case for the hexameric γCA (discussed below), and thus migrates aberrantly through gel filtration columns.

Based on studies in *Arabidopsis*, dicot βCAs may exist in two distinct compartments, namely the chloroplast and the cytosol (Fett & Coleman, 1994). Two distinct cDNAs have been cloned, and one of them appears to lack a transit peptide. Additionally, immunological evidence corroborates the existence of an extrachloroplastic βCA (Fett & Coleman, 1994). The implications of this duplication are presently unclear.

Monocot isozymes have been characterized by conceptual translation of cDNAs cloned from species such as barley, maize, and rice (Hewett-Emmett & Tashian, 1996). They are less well characterized than their dicot counterparts, but seem to demonstrate comparable reaction kinetics (Atkins et al., 1972). This class is largely distinguished...
from the dicot isozymes by a comparably lower native molecular weight, and these enzymes are thought to be dimers (Atkins et al., 1972; Graham et al., 1984). Additionally, the *Tradescantia* enzyme displays discernibly different IC$_{50}$ values for a number of inhibitors when compared to pea. For example, the pea enzyme exhibits an IC$_{50}$ for nitrate of 38 µM, while for the *Tradescantia* CA this value is 175 µM (Atkins et al., 1972).

In maize, which engages C$_4$ metabolism, two cDNAs have been cloned by Burnell and deposited in the nucleotide databases (accession numbers U08403 and U08401). As in *Arabidopsis*, it seems that one of these appears to encode a chloroplastic enzyme since it contains an amino terminus similar to transit peptides while the other gives rise to a cytoplasmic form. It can be speculated that the cytoplasmic form resides in the mesophyll to provide bicarbonate for PEP carboxylase, but this hypothesis has not been directly tested.

In higher plants, a role for the chloroplast CA is presently unknown. It has been traditionally presumed that CA would serve in a capacity similar to the isozymes of *Synechococcus* and *Chlamydomonas*. That is, chloroplast CA could serve to concentrate the availability of CO$_2$ for the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and thus reduce the metabolic costs of photorespiration. This hypothesis has been partially borne out by the observation that carbonic anhydrase levels seem to be co-regulated with Rubisco (Hudson et al., 1992). However, demonstrating this role conclusively has proved difficult.

Coleman used an antisense transgenic approach to reduce the levels of CA in tobacco by 99% (Majeau et al., 1994). However, he reported no decrease in net CO$_2$
fixation by these lines, leading one to conclude that CA does not facilitate carbon fixation. Similar results were also reported by Price et al. (1994). In contrast, Kim (1997) used the same technique to reduce the levels of CA in Arabidopsis and achieved very different results. He found that with a 90% reduction in chloroplast CA activity, plants displayed no morphological deficits when maintained on tissue culture media supplemented with sucrose. However, if sucrose was withdrawn from the media, the antisense plants either showed a marked decline or died. This lethality was furthermore shown to be rescuable in the presence of elevated levels of carbon dioxide. These results, in stark contrast to those of the Price group and the Coleman group, betray a necessary role for CA in photosynthetic carbon fixation.

The γ Carbonic Anhydrases

The γCAs are the most recently recognized members of the CA family. Presently, a γCA has only been definitively observed in a member of the Archaea, though homologous sequences and expressed sequence tags have been found in such organisms as Arabidopsis and Synechococcus (Hewett-Emmett & Tashian, 1996). The enzyme was isolated from Methanosarcina thermophila and shown to catalyze the hydration of CO$_2$ (Alber & Ferry, 1994). This isozyme, like the Chlamydomonas αCA CAH1, is secreted from the methanogen, but it is expressed in response to nutritional limitations rather than low CO$_2$ tensions. If the microbe is grown in media containing methanol, it reduces the methyl group to methane to derive metabolic energy. However, if cultures are shifted to acetate, Methanosarcina oxidizes the carbonyl group of acetate to carbon dioxide to derive energy to reduce the remaining methyl group to methane. It is this
resulting CO\textsubscript{2} that is likely acted upon by the γCA, possibly as part of a bicarbonate/acetate antiport system (Alber & Ferry, 1994).

The \textit{Methanosarcina} γCA has been crystallized to reveal a rather unusual structure (Figure 1.2; Kisker et al., 1996). The molecule, like αCA, is dominated by β strands. However, unlike αCA, the strands of γCA do not form conventional sheets, but rather form a three sided, triangular left-handed helix (Figure 1.2). The helix proceeds through seven to eight turns to form the long axis of the molecule which has the overall shape of a tube. Flanking the β helix is a single α helix which extends approximately the same length.

To form the holoenzyme, these beautiful subunits assemble into a homotrimer with parallel orientation. Based on crystallographic observations, these trimers may dimerize in a head to head fashion to form a hexamer. In this arrangement, two trimers become bridged by a β barrel formed by the six amino terminal extensions of each subunit. However, it is possible that this further oligomerization is an artifact, as sizing of γCA using the analytical ultracentrifuge indicates that it exists in solution as a trimer (Kisker et al., 1996). When viewed along the long axis of the molecule, the trimer itself forms a shape resembling a Star of David with the longitudinal α helix of each monomer forming the vertices of one triangle while the β helices form a second inverted one (Figure 1.2). The active site zinc atom is coordinated by three histidine ligands which are virtually superimposable upon the histidines of the active site of representative αCAs (Lindskog, 1997). However, unlike the αCAs, the zinc ligands of the γCA are located at the subunit interfaces of the trimer. Among the three histidine ligands at each active site,
Figure 1.2 Structure of the *Methanosarcina* γ carbonic anhydrase. A. The γCA monomer viewed perpendicular to the long axis of the β helix. B. The γCA trimer viewed down the long axis of the β helix showing the three fold symmetry of the holoenzyme. This structure was rendered with RasMol by Roger Sayle using the coordinates 1thj deposited in the Protein Data Bank by Kisker *et al.* (1996).
one donor is supplied by one monomer while the remaining two are supplied by an adjacent monomer (Kisker et al., 1996). It is thus obvious that multimeric assembly is required before the γCAs can carry out catalysis.

Overview

In the absence of a crystal structure for a βCA, structural information for this class is severely lacking. It is thus difficult to perform rational mutagenic studies to elucidate such features as the catalytic mechanism and the nature of the oligomeric assembly. Nonetheless, comparisons of primary structures of members of the β family can offer insights into the structure of these enzymes. The present work takes advantage of this approach in an attempt to gain useful information concerning the coordination of the active site zinc and the formation of quaternary structure.

References


CHAPTER 2
THE ZINC CENTER IN SPINACH CARBONIC ANHYDRASE

Introduction

Carbonic anhydrase (CA; carbonate dehydratase, EC 4.2.1.1) is a ubiquitous zinc
metalloenzyme which catalyzes the reversible hydration of carbon dioxide. In mammals, CA
plays important roles in facilitating carbon dioxide exchange in capillary beds and alveoli,
maintaining the buffering capacity of blood, and reabsorbing bicarbonate across renal tubules
(Tashian, 1989). Crystal structures for the human isozymes I and II and the bovine isozymes
II and III have been solved, and they show that these enzymes coordinate an active site zinc
through three conserved histidine residues (for example, see Håkansson et al., 1992).

In higher plants which carry out C₃ photosynthesis, the majority of CA activity can be
localized to the chloroplast stroma where the enzyme's role is unclear, although it may serve
to concentrate carbon dioxide at the active site of ribulose-1,5-bisphosphate
carboxylase/oxygenase (Graham et al., 1984). CA from higher plants is quite different from
the major mammalian isozymes in both primary sequence and multimeric assembly. CA from
C₃ dicotyledonous plants is a hexamer with one zinc atom per monomer (Graham et al.,
1984; Kisel & Graf, 1972) while the major mammalian isozymes are monomeric (Tashian,
1989). Furthermore, sequence analysis reveals no homology between the plant and animal
CA’s, and this suggests that the animal and plant isozymes do not share a common
evolutionary origin (Fukuzawa et al., 1992) and therefore may not share common physical
properties.

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Until a crystal structure becomes available for a plant-type CA, biochemical investigations may provide hints of structural characteristics of this enzyme. To this end, we have employed techniques of molecular biology and biophysics to investigate the nature of the zinc binding site in spinach carbonic anhydrase. We present here results of experiments utilizing site-directed mutagenesis, elemental analysis, and EXAFS spectroscopy.

**Experimental Procedures**

**Cloning and mutagenesis.** All DNA manipulations including plasmid isolation, restriction digestions, ligations, and transformations were performed using standard methods (Sambrook *et al.*, 1989). Site specific mutations were introduced into CA using the Altered Sites Mutagenesis System (Promega). Mutagenesis changing histidine to glutamine, cysteines to alanines, glutamates to glutamines, and aspartate to asparagine resulted in the mutants H210Q, C150A, C213A, E194Q, E266Q, and D152N. Mutations were confirmed by sequencing according to a modified dideoxy chain termination method (Fawcett & Bartlett, 1990).

Mutant constructs were cloned into a derivative of the expression vector pKK233-2 (Pharmacia LKB Biotechnology, Inc.) and expressed in *E. coli* DH5α for activity measurements. To facilitate purification, these CA's were further subcloned into a derivative of the expression vector RIT2T (Pharmacia LKB Biotechnology, Inc.) to yield constructs, termed pPAXCA, which upon translation would result in carbonic anhydrase fused to the carboxyl terminus of *Staphylococcus* protein A.

**Protein isolation and assays.** Protein was isolated from the pKK expression system for activity measurements as follows. *E. coli* DH5α harboring the appropriate CA construct
or the vector alone was grown at 37° in LB media to an OD$_{600}$ of 1.8. Cells were pelleted and resuspended in Buffer A (50 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM EDTA). After lysing by sonication, the resulting homogenate was clarified and brought to 56% saturation with solid ammonium sulfate. Protein was allowed to precipitate for at least one hour on ice and then pelleted at 32,000x$g$ for 15 minutes. The resulting pellets were resuspended and dialyzed overnight at 4° against Buffer A.

Each dialyzed sample was quantitated with the Coomassie protein assay (Pierce) using BSA as a standard. The presence of recombinant CA in each of the samples was verified by Western blotting.

Activity measurements of the crude CA preparations were determined according to the spectrophotometric method of Khalifah (1971). Briefly, 0.5 ml of an appropriate buffer-indicator pair was placed in a cuvette, followed by the addition of CA extract. The reaction was initiated by the addition of CO$_2$ saturated water, and the change in absorbance of the indicator was recorded as a function of time. We assayed in 25 mM EPPS pH 8 with 0.113 mM phenol red monitored at 555 nm and 25 mM imidazole pH 8 with 0.072 mM p-nitrophenol monitored at 400 nm. These buffer-indicator pairs were chosen based on their similar $pK_a$ values.

For each buffer system, an acid calibration curve was used to calculate the buffer factor $Q$ which was then used to convert dA/dt to mmol CO$_2$ sec$^{-1}$ mg$^{-1}$. Initial rates of CO$_2$ hydration were determined by extrapolating back to time zero from a linear sampling time. The hydration rate measured using protein derived from cells harboring the empty pKK233-2 vector was considered spontaneous and subtracted as uncatalyzed background.
Ellman's assays were performed essentially as described (Riddles et al., 1983).

**Inductively coupled plasma atomic emission spectroscopy (ICP-AES).** For the metal analysis, fusion proteins were expressed in *E. coli* and purified by affinity chromatography using non-specific IgG linked to Reacti-Gel 6x (Pierce). Purified protein A/CA fusions were freed of loosely bound zinc using Chelex 100 (Bio Rad). As negative controls, buffer alone or an amount of BSA comparable to the amount of CA fusion assayed was treated similarly. Each sample was analyzed for zinc concentration using an ARL 34000 inductively coupled plasma atomic emission spectrometer and electronics and software from Labco, Inc. For final determination of zinc binding, readings for the zinc-free BSA standard were treated as negligible background due to density and viscosity differences within the plasma torch as compared to the buffer control and were subtracted from readings for the CA fusions.

**EXAFS.** Wild-type protein A/CA fusion was purified as above from 50 g cell paste. Protein was concentrated using Minicon-B15 clinical sample concentrators (Amicon) to a final concentration of ~2 mM and supplemented with 30% ethylene glycol. An 80 μl sample was loaded into a lucite cell with a 1 mm pathlength and sealed with 0.001" Kapton tape. X-ray absorption spectra were recorded at the National Synchrotron Light Source, Brookhaven National Laboratory, on beamline X10-C. The beamline was run in focused mode with an Si(111) double crystal monochromator configuration. Higher order harmonics were rejected using a mirror position feedback system (Sansone et al., 1991). Frozen samples were loaded into an Oxford Instruments liquid helium flow cryostat maintained at ~10K. XAFS data were collected in fluorescence mode using a Canberra Industries 13-element Ge solid-state array detector (Cramer et al., 1988) while incident beam intensity was monitored with a nitrogen
filled ion chamber. Photon energy was calibrated by simultaneously collecting a transmission spectrum of a zinc metal foil and setting the first inflection point energy to be 9659.0 eV.

EXAFS oscillations were extracted from the raw data by routine methods (Cramer et al., 1978) and were then quantitatively analyzed using a Levenberg-Marquadt nonlinear least-squares calculated curve-fitting procedure to minimize differences between the data and observed EXAFS. Simulations were derived from the curved-wave functional form:

\[ \chi(k) = \sum_i N_i \gamma_i f_i(k, R_i) \frac{k R_i^2}{e^{-2 \sigma^2 k^2}} \sin[2kR_i + \phi_i(k, R_i)] \]

(McKale et al., 1986). For this analysis, theoretical values for both phase and amplitude were used, and the value \( g \) was fixed at 0.9 for all fits. During fitting, the total Zn coordination was set to four or five and small changes in the threshold energy (DEo) were fixed at -4.2 eV (Hubbard et al., 1991), while the interatomic distance (R) and the mean square deviation of R (s²) were allowed to vary.

Results

Sequences for the *E. coli* CA homologue (Sung & Fuchs, 1988), the *Synechococcus* CA (Fukuzawa et al., 1992), and CA's from *Arabidopsis* (Raines et al., 1992), pea (Roeske & Ogren, 1990), spinach (Fawcett et al., 1990), tobacco (Majeau & Coleman, 1992), and barley were analyzed for conservation of the amino acids whose side chains are known to serve as zinc ligands at enzyme active sites (Vallee & Auld, 1990). Homology alignments showed that one histidine, two cysteines, two glutamates, and one aspartate are conserved among the plant-type CA's (Figure 2.1). Mutagenesis changing these six residues resulted in mutant forms which displayed altered catalytic activity (Table 2.1). Western blotting of
Figure 2.1 Homology among the plant and bacterial carbonic anhydrases. Alignment is shown of the portion of the plant-type CA’s spanning the six conserved potential zinc ligands from spinach, pea, tobacco, Arabidopsis, E. coli, and Synechococcus. The alignment begins with amino acid 149 in the spinach sequence as deposited in GenBank. Asterisks indicate potential ligands to zinc. Bold asterisks indicate potential zinc ligands that, when mutated, resulted in CA that bound zinc poorly. Alignment generated by PILEUP of the UWGCG programs.
Table 2.1 Activity of and zinc binding to mutants of spinach carbonic anhydrase.

<table>
<thead>
<tr>
<th>MUTANT</th>
<th>ACTIVITY</th>
<th>EPPS</th>
<th>imidazole</th>
<th>mol Zn / mol CA monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>100</td>
<td>100</td>
<td>1.09</td>
</tr>
<tr>
<td>C150A</td>
<td>ND</td>
<td>9</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>D152N</td>
<td>1</td>
<td>9</td>
<td></td>
<td>0.81</td>
</tr>
<tr>
<td>E194Q</td>
<td>22</td>
<td>5</td>
<td></td>
<td>0.80</td>
</tr>
<tr>
<td>H210Q</td>
<td>10</td>
<td>6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C213A</td>
<td>6</td>
<td>5</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>E266Q</td>
<td>81</td>
<td>100</td>
<td></td>
<td>1.25</td>
</tr>
</tbody>
</table>

Activities are expressed as percentages of the wild-type enzyme in each buffer system. The blank-corrected activity of the wild-type was 6.8 mmol CO₂ sec⁻¹ mg⁻¹ E. coli protein in EPPS buffer and 76 mmol CO₂ sec⁻¹ mg⁻¹ E. coli protein in imidazole. Reported activities represent an average of four to five independent assays from two preparations. The assay was limited by an uncatalyzed rate of hydration of 7.9 mmol CO₂ sec⁻¹ mg⁻¹ E. coli protein in EPPS buffer and 32 mmol CO₂ sec⁻¹ mg⁻¹ E. coli protein in imidazole. ND, Not detectable.
lysates from *E. coli* expressing each of the mutant proteins revealed that all were expressed in soluble form. (Data not shown.)

The six spinach CA mutants and the wild-type enzyme were also analyzed for their ability to bind zinc. In order to facilitate purification of the proteins, the mutant forms as well as wild-type CA’s were expressed in *E. coli* as C-terminal fusions to protein A. The wild-type CA fusion protein was catalytically active, indicating that the protein A moiety does not significantly interfere with proper folding or function of the CA portion of the fusion. After purification, the fusion proteins were depleted of adventitious zinc and analyzed by inductively coupled plasma atomic emission spectroscopy. Wild-type CA bound zinc in a ratio of one atom per subunit. However, the mutants C150A, H210Q, and C213A, had greatly diminished capacities to bind zinc (Table 2.1).

Two of the latter mutant CA’s were more susceptible to proteolysis than the wild-type protein when expressed in *E. coli*, and during affinity chromatography various breakdown products co-purify. These breakdown products are evident in Figure 2.2 and are shown with the wild-type enzyme and two stable mutants for comparison. These products likely represent various C-terminal truncations that are still able to interact with the IgG column through the protein A domain at the amino terminus. Regardless, even if intact fusion represented only 35% of the total protein assayed in the case of C150A and only 10% in the case of H210Q, ratios of zinc to CA monomer would still be less than 0.3 based on back-calculation from the ICP-AES data.

The instability of the cysteine mutant C150A is not due to the disruption of a disulfide bond involving this residue. Ellman’s assays of the wild-type fusion protein revealed the
Figure 2.2 Proteolytic susceptibility of selected mutant CAs. SDS-PAGE of the purified CA fusions assayed for zinc content showing the instability of two mutant proteins that bind zinc poorly. Lane 1, WT; lane 2, H199Q; lane 3, H210Q; lane 4, C150A; lane 5, C213A. Molecular weight markers are indicated. The arrowhead at right indicates the protein A/CA fusion bands.
presence of two reduced cysteines per monomer in the native state and all six cysteines per monomer in the presence of 6M guanidinium chloride. This indicates that no disulfides exist in the bacterially expressed spinach CA, though four of the six cysteines present per monomer are inaccessible to solvent in the native state.

To further investigate the nature of the ligands in the wild-type CA fusion protein, we examined its Zn X-ray absorption spectrum. The fluorescence-detected Zn K-edge spectrum is shown in Figure 2.3. The CA Zn EXAFS is relatively strong and without a clear beat pattern (Figure 2.4A). The Fourier transform is dominated by a single peak centered at ~2.3 Å with a few minor peaks above the noise between 3-4 Å (Figure 2.4B). The pattern is similar to the transforms observed in EXAFS studies of plastocyanin and other blue Cu proteins (Scott et al., 1982).

The dominant feature could be simulated by a Zn-S interaction at ~2.3 Å, but additional Zn-N/O interactions near 2 Å were necessary to get a good fit. The small features present beyond the central 2.3 Å peak are most probably due to multiple scattering interactions from an imidazole group and some contributions from the carbons of the cysteines. Unfortunately, with little knowledge of the symmetry and geometry of the site, attempts to fit these features with multiple scattering have not been successful. The quality of fit for all possible combinations of sulfur and nitrogen/oxygen interactions was judged by comparing the fit index between the resulting fit and the raw extracted EXAFS, and these results are summarized in Table 2.2.
Figure 2.3 Zn K-edge XAS spectrum. Pre-edge subtracted absorption spectrum of the protein A/CA fusion.
Figure 2.4 Results of EXAFS curve fitting analysis. A. Experimental $k^2$ EXAFS (solid line) and final fit results for 2 Zn-S -- 2 Zn-N/O coordination to Zn (dashed line). B. Fourier transform of EXAFS (solid line) with final fit results (dashed line).
Table 2.2 Results of EXAFS fitting analysis.

<table>
<thead>
<tr>
<th>Model</th>
<th>N</th>
<th>R (Å)</th>
<th>(\sigma^2) (Å² (\times 10^3))</th>
<th>Fit Index (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetracoordinate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn-S</td>
<td>4</td>
<td>2.31</td>
<td>6.29</td>
<td>314</td>
</tr>
<tr>
<td>Zn-S</td>
<td>3</td>
<td>2.32</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>Zn-N/O</td>
<td>1</td>
<td>2.04</td>
<td>1.35</td>
<td>212</td>
</tr>
<tr>
<td>Zn-S</td>
<td>2</td>
<td>2.32</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>Zn-N/O</td>
<td>2</td>
<td>2.06</td>
<td>3.02</td>
<td>171</td>
</tr>
<tr>
<td>Zn-S</td>
<td>1</td>
<td>2.32</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Zn-N/O</td>
<td>3</td>
<td>2.08</td>
<td>5.09</td>
<td>255</td>
</tr>
<tr>
<td>Zn-N/O</td>
<td>4</td>
<td>2.11</td>
<td>4.90</td>
<td>777</td>
</tr>
<tr>
<td><strong>Pentacoordinate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn-S</td>
<td>2</td>
<td>2.32</td>
<td>2.37</td>
<td></td>
</tr>
<tr>
<td>Zn-N/O</td>
<td>3</td>
<td>2.06</td>
<td>5.63</td>
<td>143</td>
</tr>
</tbody>
</table>

\[ F = \left[ \frac{\Sigma (\chi_e - \chi_o)^2 k^4}{n} \right] \]

where the difference is between each data point of the experimental (\(\chi_o\)) and simulated (\(\chi_e\)) EXAFS, and \(n\) is the number of data points in the fitting range. All fits performed on range \(k = 2-13\ \text{Å}^{-1}\). During fitting, the total Zn coordination was set to four or five and small changes in the threshold energy (\(\Delta E_o\)) were fixed at -4.2 eV (Hubbard et al., 1991), while the interatomic distance (R) and the mean square deviation of R (\(\sigma^2\)) were allowed to vary.

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Discussion

The plant-type carbonic anhydrases have long been overshadowed by the animal isozymes, but the former are emerging as rather intriguing enzymes in their own right. Though it is unlikely that the animal and plant-type CA’s are evolutionarily related (Fukuzawa et al., 1992), these two groups share many common features. Both are zinc metalloenzymes which catalyze the hydration of carbon dioxide with remarkable efficiency and are susceptible to a number of common inhibitors. Among these, the sulfonamides inhibit the animal isoforms by binding to the catalytic zinc (Vidgren et al., 1990). By analogy, since sulfonamides also inhibit plant-type CA’s (Pocker & Ng, 1974) it is presumed that the zinc bound to the plant enzyme is also catalytic in nature. However, in contrast to the animal enzyme, plant CA’s do not possess three conserved histidines to account for zinc coordination consistent with the animal model. Therefore, the two isoforms may have evolved to coordinate the active site metal by two very different strategies.

To investigate how spinach CA might coordinate zinc, we analyzed available sequences of plant-type CA’s for the conservation of all amino acids whose side chains are known to serve as ligands to active site zincks, namely histidine, aspartate, glutamate, and cysteine (Vallee & Auld, 1990). Six such conservations were identified and each residue was targeted for mutagenesis (Figure 2.1). Among these, the aspartate, histidine, and cysteine mutants each exhibited less than 10% of the activity of the wild-type enzyme when assayed in EPPS buffer (Table 2.1).

Mutations in the carbonic anhydrase from pea at locations equivalent to those described here result in similar enzyme performance (Provart et al., 1993). Pea mutants
corresponding to the spinach H210Q, C150A, and C213A (H220N, C160S, and C223S, respectively) had no measurable activity, reinforcing our results with the spinach enzyme. Additionally, Coleman's group found the pea E204A to exhibit no activity and E276A to be rather compromised. However, we find the equivalent, more conservative spinach mutant E266Q to display near wild-type activity, while E194Q is 22% as active as the wild-type enzyme. We attribute these differences of results to Coleman's more severe charged to aliphatic mutations which may serve to disrupt the pea enzyme more than the conservative spinach mutation. Since Coleman's group did not construct a mutation comparable to D152N, we are unable to compare our results regarding this variant. Nonetheless, a duplication of enzymatic trends among various mutants from two species provides further evidence that H210, C150, and C213 are critical residues in spinach CA.

In previous studies on other zinc proteins, site-directed mutagenesis of potential zinc ligands followed by the loss of function of the metalloprotein in question has been provided as evidence implicating residues in metal coordination. This has been demonstrated for leukotriene A4 hydrolase (Medina et al., 1991), neutral endopeptidase (LeMoual et al., 1993), and a potential zinc finger in the glucocorticoid receptor (Severne et al., 1988). We have shown here the same correlation in spinach CA; of six conserved potential zinc ligands, four can be mutated to cause a 90% loss of function (Table 2.1). As the zinc of CA is presumably catalytic in nature, the loss of the enzyme's ability to coordinate this metal would necessarily result in a concomitant loss of activity. Thus, based on our activity assays, the conserved histidine, cysteines, and aspartate are all candidates for zinc ligands in spinach carbonic anhydrase.
Additionally, we have definitively ascertained which of these mutations affect the enzyme's ability to bind zinc rather than affect catalysis through some secondary disruption. Such a disruption could conceivably occur by various means, for example by abolishing a hydrogen bond network within the active site as suggested for the pea mutant E276A (Provart et al., 1993). Through elemental analysis we have established that the histidine and cysteine mutants uniquely exhibit severely diminished capacities to bind zinc (Table 2.1). This correlation between a loss of activity and loss of zinc binding has also been shown for the zinc ligand mutant H94D in human CA II (Kiefer et al., 1993) and mutants of leukotriene A₄ hydrolase (Medina et al., 1991). We interpret this as additional proof indicating that these three residues are involved in the coordination of the zinc ion, while the aspartate may play an essential role unrelated to metal coordination.

While the ICP-AES data do not implicate the glutamate and aspartate mutants as potential zinc ligands, these variants do demonstrate significant catalytic disruptions. To investigate if these residues might be involved in a proton transfer step within the active site as suggested for various amino acids in the animal isozymes, we also assayed in an imidazole buffer system. As a relatively small buffering molecule, imidazole is thought to complement the catalytic deficiency of proton shuttle mutants by entering the active site and essentially replacing the missing functional group. This property has been exploited in the animal systems to identify proton shuttling amino acids such as R67 in human CA III (Tu et al., 1990). We find that imidazole does indeed restore the activity of E266Q to wild-type levels, implicating this residue as a possible proton shuttle in the active site. This conclusion has also been reached for the equivalent pea CA mutation (Provart et al., 1993). The remaining

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mutants do not exhibit a stimulatory imidazole effect. It is interesting that the activity of E194Q actually decreases relative to the wild-type enzyme in the imidazole system (Table 2.1). The reasons for this decline are not immediately obvious.

An increased susceptibility to endogenous *E. coli* proteases during the purification of C150A and H210Q is demonstrated in Figure 2.2, and this may represent an instability of the mutant proteins. Similar instability has been documented in other systems where zinc metalloenzymes have been depleted of zinc and assayed for resistance to trypsin proteolysis. In the case of the DNA binding domain of the GAL4 transcription factor, the zinc-free apoenzyme was reduced to peptides upon trypsinization while the zinc-bound form was cleaved to a 13 kDa core particle (Pan & Coleman, 1989). Similar results were also obtained with the Gene 32 protein from bacteriophage T4 (Giedroc et al., 1987). Furthermore, differential scanning calorimetry investigations of the folding processes of the Gene 32 protein as well as alkaline phosphatase from *E. coli* showed metal dependent changes in the denaturation profiles of these zinc metalloproteins (Keating et al., 1988; Chlebowski & Mabrey, 1977). These results imply that the binding of the metal imparts significant stabilization to these proteins at the level of tertiary structure, or, as suggested for aspartate carbamoyltransferase, at the level of quaternary structure (Monaco et al., 1978).

These characteristics may also hold true for spinach carbonic anhydrase. Possibly, the formation of the active site is necessary for the proper folding of CA monomers; the inability to bind the metal could disturb elements of the protein's secondary or tertiary structure, exposing protease sensitive domains. Alternatively, the active sites of spinach CA may be located at subunit interfaces and zinc could stabilize the quaternary structure of the
holoenzyme. Though the zinc of CA is presumably catalytic in nature, this established correlation of protein stability and metal binding is a possible explanation for the susceptibility to proteolysis in our CA mutant proteins.

To further investigate the zinc binding site in wild-type spinach carbonic anhydrase, we analyzed this enzyme's extended X-ray absorption fine structure. EXAFS has the advantage of providing a direct examination of a metal's environment in metalloproteins. Analysis of the spectrum of spinach CA yields evidence that the nearest neighbors of the zinc are sulfur and nitrogen/oxygen species. Of the five possible combinations of two distinct Zn-S and Zn-N/O interactions in a four ligand system, 2 Zn-S - 2 Zn-N/O gives the best fit, in agreement with the results of the mutagenesis. This model represents a 20% improvement in fit index over the next lowest value (Table 2.2). It is clear that a model with four N/O ligands coordinated to Zn, as found in mammalian CA, is not consistent with the EXAFS since the corresponding fit is of much worse quality than the others performed on this system. Attempts to fit a longer N/O shell at ~2.3 Å were also unsuccessful, yielding fit indices ~10^{-4} or causing the second Zn-N/O distance to contract back to ~2.1 Å, giving the same result as the 4 N/O model. Furthermore, the presence of only two conserved cysteines per polypeptide in the plant-type CA's is consistent with our 2 Zn-S - 2 Zn-N/O EXAFS model and renders the 3 Zn-S - 1 Zn-N/O and 4 Zn-S - 0 Zn-N/O models improbable.

Since four separate CA mutants yielded enzymes with activities of 10% or less, we also examined the EXAFS data for the possibility of a pentacoordinate zinc as implicated for adenosine deaminase (Wilson et al., 1991; Bhaumik et al., 1993). The fit index resulting from the addition of a third N/O ligand at ~2.0 Å for a 2 Zn-S - 3 Zn-N/O system does yield a
16% improvement over the 2 Zn-S - 2 Zn-N/O model. However, several lines of evidence discredit this five ligand model. First, the fit indices for the best tetracoordinate model and the pentacoordinate model lie within the usual error range placed on values of coordination derived using the EXAFS technique. Therefore, it is objectively impossible to favor one model or the other based purely on EXAFS results. Second, when a tetracoordinate zinc is converted to a pentacoordinate one in a synthetic system, the bond length of the leaving group is longer than the four remaining ligand bond lengths (Auf der Heyde & Nassimbeni, 1984). We can find no such asymmetry in the CA pentacoordinate model; all N/O bond lengths remain the same. Third, only three of our potential ligand mutants exhibit a diminished capacity for zinc binding. If we define a ligand as a residue that contributes to the binding of a metal, then the EXAFS data may indicate that D152 is a near neighbor of the metal, while the ICP-AES data indicate that this residue is not a ligand per se. Overall, we find the tetracoordinate system to be a more credible model.

Monozinc enzymes may be classified into families based on the spacing of zinc ligands and the conservation of amino acids adjacent to them (Vallee & Auld, 1990). A typical catalytic zinc-binding site in this nomenclature is made up of two closely spaced ligands L1 and L2, which comprise a zinc binding nucleus separated by an amino acid spacer X, plus a third, distant L3 separated by a spacer Y. In the case of spinach CA, H210 and C213 may represent L1 and L2, respectively, while C150 may represent L3, with X=2 and Y=59. A preliminary search of the SwissProt database for homology with the amino acid motif encompassing the L1-L2 nucleus revealed no significant homology with any known zinc...
metalloenzymes. Thus, the plant-type carbonic anhydrases may represent a novel family of zinc metalloproteins.

In summary, site-directed mutagenesis of potential zinc ligands in spinach carbonic anhydrase revealed that mutant proteins in which cysteine 150 was converted to alanine, aspartate 152 was converted to asparagine, histidine 210 was converted to glutamine, or cysteine 213 was converted to alanine exhibited severely diminished catalytic activity. Furthermore, the cysteine and histidine mutants had greatly diminished capacities to bind zinc. In addition, EXAFS data are consistent with a proposed metal coordination by two sulfurs, while the bond lengths suggest tetracoordination, presumably by additional nitrogen or oxygen donor ligands. The residues corresponding to cysteine 150, histidine 210, and cysteine 213 are conserved in all plant-type CA's sequenced to date, and so our results suggest that the side chains of these amino acids are the protein ligands to the active site Zn, while a reactive water molecule may be inferred to complete the coordination sphere. With this ligand scheme, spinach CA joins alcohol dehydrogenase and cytidine deaminase as the third zinc metalloenzyme in which a catalytic zinc is coordinated by sulfur ligands (Vallee & Auld, 1990; Betts et al., 1994). Though a definitive resolution of the role of the conserved aspartate will await the elucidation of a CA crystal structure or a detailed enzymological study of D152 mutants, the plant-type CA's seem to differ from the mammalian isozymes not only in primary sequence and quaternary structure, but also in the nature of the side chains responsible for binding the catalytic zinc.

References


CHAPTER 3
SEQUENCE OF A cDNA ENCODING
CARBONIC ANHYDRASE FROM BARLEY

Introduction

Carbonic anhydrase (CA) catalyzes the reversible hydration of CO₂ to generate bicarbonate and a proton. Though its physiological function has not been clearly defined, it had been speculated that CA serves to concentrate CO₂ at the active site of ribulose-1,5-bisphosphate carboxylase/oxygenase (Graham et al., 1984). However, recent work with transgenic plants expressing CA in the antisense orientation has shown that the enzyme does not play an essential role in carbon fixation, though the regulation of CA levels seems to parallel that of carboxylase (Majeau and Coleman, 1994; Majeau et al., 1994; Price et al., 1994). Currently, several sequences are available for CAs from C₃ dicots (for a recent listing, see Bracey et al., 1994 and references therein); we present here what we believe to be the first available sequence of a CA from a C₃ monocot.

Results and Discussion

The cDNA that we have isolated is 1530 base pairs long (Figure 3.1). Its essential features are summarized in Table 3.1. Based on a comparison with the dicot CAs, we predict that the barley clone contains 246 base pairs of 5' non-translated sequence and 311 base pairs of 3' non-translated sequence. Interestingly, we isolated two phage clones harboring identical CA inserts with different polyadenylation sites, one beginning at base 1516 and one beginning at base 1505. A potential polyadenylation sequence, AATATAA, occurs at base 1472.

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Figure 3.1 Nucleotide sequence of barley carbonic anhydrase cDNA, NCBI accession number L36959. The predicted translation start and stop sites are shown in bold at positions 247 and 1219, respectively.
Table 3.1 Characteristics of the CA cDNA from barley.

<table>
<thead>
<tr>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species:</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em> L.</td>
</tr>
<tr>
<td>Gene:</td>
</tr>
<tr>
<td>Chromosomal location unknown</td>
</tr>
<tr>
<td>Subcellular location:</td>
</tr>
<tr>
<td>Chloroplast stroma</td>
</tr>
<tr>
<td>Predicted DNA fragments:</td>
</tr>
<tr>
<td>5' flanking region, 246 bp; coding region, 972 bp; 3' flanking region, 311 bp</td>
</tr>
<tr>
<td>Techniques:</td>
</tr>
<tr>
<td>λgt10 cDNA library (Ling and Zielinski, 1989) screening using spinach CA cDNA</td>
</tr>
<tr>
<td>(Fawcett et al., 1990), cloning into pBluescript vector (Stratagene), restriction</td>
</tr>
<tr>
<td>fragment subcloning, and modified Sequenase v2.0 (USB) sequencing of both</td>
</tr>
<tr>
<td>strands (Fawcett and Bartlett, 1990)</td>
</tr>
<tr>
<td>Predicted features of the protein:</td>
</tr>
<tr>
<td>The deduced protein sequence consists of 324 amino acids with a calculated</td>
</tr>
<tr>
<td>molecular weight of 35,074. The amino terminus is rich in hydroxylated and</td>
</tr>
<tr>
<td>positively charged amino acids which likely confer chloroplast localization.</td>
</tr>
</tbody>
</table>
In the past, comparisons of CAs of both monocot and dicot origin suggested that these two families would be quite different. The two classes have different quaternary structure, respond differently to inhibitors, exhibit different stability, and display different reaction kinetics (Atkins et al., 1972). Despite these observations, the deduced protein sequence of barley CA bears remarkable resemblance to the dicot isoforms. When barley CA was compared to spinach CA using the GCG comparison program BESTFIT, the predicted mature proteins exhibited 60% identity and 74% similarity if conservative substitutions are taken into account.

One immediately obvious difference between the inferred barley protein sequence and the dicot CAs is an apparent truncation at the monocot carboxyl terminus, which lacks a conserved ten amino acid “tail” present in all dicots so far examined. We are presently examining this region to investigate its possible role in higher oligomerization states among the dicot isoforms.

References


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CHAPTER 4
A DETERMINANT OF QUATERNARY STRUCTURE IN SPINACH CARBONIC ANHYDRASE

Introduction

Unlike the majority of α carbonic anhydrases, the β forms exist as oligomers (Graham et al., 1984). The oligomeric states of a number of βCAs have been investigated over the years, and differing results have been reported concerning their exact quaternary structure. For example, dicot CAs have been reported as both hexamers and as octamers when examined by different groups using different techniques (Graham et al., 1984). It is currently unclear whether this discrepancy reflects a misinterpretation of data, an in vitro artifact, or a bona fide plasticity of the oligomerization process. One possible explanation is that CA assumes a non-ideal three dimensional shape which results in aberrant migration through gel filtration columns. Accordingly, results obtained by equilibrium sedimentation or cross-linking may be the most reliable estimates of CA's exact quaternary structure, and data from the latter technique suggest that the native enzyme is an octamer (Björkbacka et al., 1997). Thus, apparent hexamers that elute from gel filtration columns are likely, in fact, octamers. Nonetheless, gel filtration is still useful for determining the relative populations of different oligomeric states.

One observation concerning the oligomerization of βCAs that is agreed upon, however, is the fact that isozymes derived from dicots differ markedly from those derived from monocots. For example, the βCAs from spinach, pea, and parsley have all been reported as hexamers or octamers while the βCAs from barley, wheat, and
*Tradescantia* have been reported as trimers or dimers (Graham *et al.*, 1984; Tobin, 1970; Atkins *et al.*, 1972). This distinction between these two closely related families of the βCAs led us to examine their primary structures for possible motifs which might explain this difference in multimeric assembly. One obvious difference between the monocot and dicot isozymes is the apparent truncation in the monocot forms of a ten amino acid carboxy terminal extension. We first observed this difference upon characterizing the barley cDNA, which at the time was the only available monocot sequence (Chapter 3). As additional monocot sequence information became available for CAs from rice (accession number U08404) and maize (U08403, U08401), it became clear that this distinction was not a feature unique to barley. In the studies reported here, we examined the effects of deletion of this "tail" on the oligomeric state of the spinach enzyme. Further, we showed that the carboxyl terminus can mediate protein-protein interactions within the βCA oligomer and propose a speculative model that unifies our observations and those of Björbacka *et al.* (1997).

**Experimental Procedures**

Cloning and mutagenesis. All DNA manipulations, cloning, and expression of proteins were performed using standard methods (Sambrook *et al.*, 1989; Bracey *et al.*, 1994). Site directed mutations were introduced into spinach carbonic anhydrase using the Altered Sites Mutagenesis System (Promega). The mutant C213A alters an inferred zinc ligand and has been described previously (Bracey *et al.*, 1994; Chapter 2). The mutant E310A was generated by engineering an ochre stop codon in the place of glutamate at amino acid 310, resulting in a carboxy terminal truncation lacking ten amino acids. Qualitatively, this truncation did not alter catalytic activity.
To analyze protein-protein interactions among the various CA constructs, we subcloned appropriate fragments into the two-hybrid vectors pAS2-l and pACT2 (Clontech). Constructs expressing the full length mature spinach CA from an engineered NcoI site at amino acid 78 were cloned into the two-hybrid vectors to yield the constructs pAS-CA and pACT-CA. To accomplish this, pPAxCA (Chapter 2) was digested with NcoI and NsiI, and the resulting insert was cloned into the NcoI-PstI sites of pAS2-l to yield pAS-CA. Additionally, pKKCA78 (Fawcett et al. 1990) was digested with NcoI and EcoRI and the resulting insert was ligated into the corresponding sites in pACT2 to generate pACT-CA. The two mutants, E310Δ and C213A, were cloned into the two-hybrid vectors by starting with constructs in the Pharmacia vector pKK-233. These plasmids were digested with NcoI and NsiI and the resulting inserts were either cloned directly into the NcoI-PstI sites in pAS2-l or into the NcoI-PstI sites of LITMUS29 (New England Biolabs). The LITMUS constructs were then digested with NcoI and EcoRI and the resulting restriction fragment ligated into the corresponding sites of pACT2. This cloning strategy generated the constructs pAS-E310Δ, pACT- E310Δ, pAS-C213A, and pACT-C213A. To express the carboxy terminal domain alone as GAL4 two-hybrid fusions, an NcoI site was introduced at amino acid 309 using the Altered Sites Mutagenesis System and the vector pSelect (Promega). This mutant was then digested with NcoI and EcoRI, and the resulting insert was subcloned into both pAS2-l and pACT2 digested with the same restriction enzymes. This strategy resulted in the expression of a fragment encoding the ten amino acid carboxy terminus of CA in the two-hybrid vectors to yield the fusion constructs pAS-tail
and pACT-tail. A summary of these constructs is depicted in Figure 4.1 where the 5' end contains the Ncol cloning sites.

**Two-hybrid experiments.** To assay interactions of the various CA proteins, mutants, and domains, cDNAs subcloned into the yeast two-hybrid vectors pAS2-1 and pACT2 were transformed in different combinations into the yeast strain Y190. Transformants were plated on complete synthetic media lacking both tryptophan and leucine supplemented with glucose and grown at 30 degrees until colonies were visible. Colonies were then picked and inoculated into liquid media of the same formulation and grown out for two days. To assay for β galactosidase activity, one milliliter of culture was pelleted in a microfuge tube, rinsed with buffer A (100 mM sodium phosphate, pH 7; 10 mM KCl; 1 mM MgSO₄), and then resuspended in buffer A with 40 mM β-mercaptoethanol. The resuspended pellet was subjected to five rounds of freeze thaw by sequentially placing the tube first in liquid nitrogen and then in a 37 °C water bath. The assay was initiated by addition of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) to a final concentration of 1 mg/ml. After a period of approximately sixteen hours at 30 °C, development of the blue reaction product was visually verified and compared to both positive and negative controls.

**Gel filtration chromatography.** Relative molecular weights were determined for both spinach CA and the E310A mutant by gel filtration chromatography using Sephacryl S200 (Sigma). The two CAs were each expressed in *E. coli* from a derivative of the expression vector pKK-233 (Pharmacia), and total clarified lysates were applied to a calibrated column. Molecular weight standards used to calibrate the column included sweet potato β-amylase (200 kDa), bovine serum albumin (66 kDa), bovine carbonic
Figure 4.1 Summary of carbonic anhydrase constructs used to make the two-hybrid fusions.
anhydrase II (29 kDa), and equine cytochrome c (12.4 kDa). Peak fractions were collected which corresponded to the molecular weights of hexamers, dimers, and monomers, and these samples were applied to SDS-PAGE gels for Western analysis using an anti-CA polyclonal antibody (Fawcett et al., 1990).

**Results and Discussion**

An investigation of the primary structure of available plant βCAs revealed a distinct difference between the monocot and the dicot forms (Figure 4.2). The latter group retains a ten amino acid “tail” at the carboxyl terminus of the protein which is missing in the monocot isozymes. We have examined this domain for its potential to mediate the higher order oligomerization characteristic of the dicot βCAs which contrasts with the oligomerization states reported for the monocot isozymes (Graham et al., 1984).

Both the wild type spinach CA and a corresponding carboxyl truncation were expressed in *E. coli* and analyzed for their multimeric assembly by gel filtration. The wild type enzyme migrated through the column with an apparent relative molecular mass consistent with hexamers, though this population is presumably octameric for reasons discussed above (Figure 4.3). We also detected a faint band in samples collected from fractions corresponding to dimers, though we cannot rule out the possibility that this is a trail of the hexamer peak. We do note that this bimodal profile was previously observed for spinach CA and explained to be the result of an assembly equilibrium in which both dimeric and hexameric states are present. It was further shown that this dissociation is not irreversible since the dimers could be concentrated to reform a hexameric species (Pocker and Miksch, 1978).
Figure 4.2 Carboxy terminal sequence alignment of representative plant carbonic anhydrases. Asterisks indicate a hydrophobic heptad repeat. The boldface E indicates the glutamate in spinach CA which was mutated to yield either the truncated E310Δ mutant or the Ncol site used to subclone the carboxyl terminus. Sequences are shown for two species of Flaveria (U08402, U08402), Arabidopsis thaliana (L18901), tobacco (M94135), spinach (J05403), pea (X52558), rice (U08404), barley (L36959), and maize (U08401). Alignment was generated using PILEUP of the Wisconsin GCG sequence analysis suite. Dicots are underlined.
Figure 4.3 Gel filtration sizing of spinach carbonic anhydrase and the E310Δ mutant. Peak fractions corresponding to hexamers, dimers, and monomers were collected, pooled, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was then blotted to nitrocellulose and probed with a rabbit polyclonal antibody directed against spinach CA. Immunoreactive bands were visualized using a horse radish peroxidase-coupled secondary antibody and luminol. T, total lysate; H, apparent hexamers; D, apparent dimers; M, monomers; WT, wild type.
Conversely, the E310Δ mutant eluted from the column at a relative molecular weight corresponding to dimers with a faint signal also present in the hexamer fraction (Figure 4.3). The band observed in samples collected from the higher molecular weight fraction appears to be a genuine tail belonging to the dimeric peak based on careful inspection of fractions intermediate between the two molecular weights. Thus, elimination of the carboxyl terminus has rendered CA incapable of forming the higher molecular weight oligomeric species. We therefore propose that the carboxyl terminus contributes to a stabilizing interface which mediates either the dimer to tetramer or the tetramer to octamer transition. Presently, we cannot say whether the E310Δ mutant is a dimer or a tetramer, so a definitive verification of its stoichiometry will await a more quantitative investigation of this assembly. Regardless, we have clearly shown that the tail contributes to an oligomerization step.

To demonstrate that it is, in fact, the tail itself which mediates this interaction and not that its deletion causes a secondary effect responsible for the disruption, we pursued studies of CA’s assembly using the two-hybrid system. With this method, we were able to express the wild type enzyme, two different mutants, and the carboxyl terminus in various combinations. The results of this approach are summarized in Table 4.1. We find that this system demonstrates the assembly of the wild type spinach CA as well as the point mutant C213A. We cannot say, however, whether the observed assembly is at the level of the dimer, tetramer, or octamer, but only that these two-hybrid fusion proteins will at least dimerize in vivo. Curiously, we cannot detect the interaction between E310Δ subunits, which we know to be at least dimeric based on the gel
Table 4.1 Interactions of various CA constructs assayed in the two-hybrid system.

<table>
<thead>
<tr>
<th>binding domain</th>
<th>activation domain</th>
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<td>CA</td>
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<td>CA</td>
<td>+</td>
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<tr>
<td>E310Δ</td>
<td>-</td>
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<tr>
<td>C213A</td>
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<td>TAIL</td>
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β-galactosidase activity is expressed as either detectable (+) or not detectable (-). *This interaction was of significantly weaker strength than the other positive signals.
filtration data. The reasons for this are potentially explained by our model for CA assembly (discussed below), and it may be that the two-hybrid data reflects the assembly of tetramers into octamers.

When the carboxyl terminus was co-expressed with the wild type CA, no interaction was observed. However, we could demonstrate an interaction between the carboxyl terminus and C213A (Table 4.1). The reasons for this difference are not clear, but the interaction of C213A with the tail does demonstrate that the carboxyl terminus is capable of mediating intermolecular assembly.

Two possibilities exist to explain how the carboxyl terminus might mediate CA's oligomerization. The tail may contain specific amino acids which are involved in discreet interactions at subunit interfaces. Indeed, this very role has been shown for the carboxyl terminus in the enzyme nucleoside diphosphate (NDP) kinase (Mesniladrey et al., 1998). Like CA, NDP kinase is found in two different oligomeric states in different phylogenetic groups. In prokaryotes, the enzyme is a tetramer while in eukaryotic organisms the enzyme forms a hexamer, and this difference correlates with the presence or absence of a five amino acid carboxyl extension which is found only in the eukaryotic proteins. In the crystal structure of this enzyme, the carboxy terminal glutamate hydrogen bonds to aspartate 115 in the neighboring subunit at the trimer interface within the hexameric enzyme. Dicot βCAs may have adopted an analogous mechanism for mediating higher order assembly via the carboxyl extension.

Alternately, the tail may form a structural feature which interacts with domains on neighboring subunits. If the tail forms a β strand that is included in an intersubunit sheet or barrel, it would display several amino acids capable of hydrogen bonding.
including E310, S314, and S318. On the other hand, if the tail forms an α helix, not only could these interactions possibly stabilize its structure, but a potential hydrophobic heptad repeat could form a coiled coil (Figure 4.2).

Of these two possibilities, our two-hybrid data suggest that the tail forms some structural element which interacts with other CA subunits since one would not expect to see a two-hybrid interaction as a result of a single amino acid hydrogen bond or salt bridge. However, this model is discredited by the observation that the Self Optimized Prediction Method, which is a collection of secondary structure prediction algorithms, suggests that the carboxyl terminus of spinach CA is largely unstructured (Geourjon & Deleage, 1994). A definitive demonstration of either of these possibilities will likely await the elucidation of a crystal structure for this enzyme.

Concerning the quaternary structure of the enzyme, we have devised a highly speculative model for CA's oligomerization which is both testable and consistent with available data. Aliev et al. (1986) proposed that CA forms a double donut shape with 422 symmetry, and we can build a model of CA based on this symmetry that accounts for available data on the subject. Björkbacka et al. (1997) were able to use cross linking agents to isolate monomers, dimers, and tetramers of a tetrameric mutant of pea CA, but they were unable to isolate trimers. If we try to reconcile this observation with Aliev’s symmetry, the two are consistent with a tetramer that has four fold rotational symmetry and which is built from monomers which each have two different reactive sites for the cross linker used. If the monomers each possessed two equally reactive sites, the cross linker would have equal opportunity to bridge any given interface and thus should produce trimers in a statistically predictable amount. This is not the case. What was observed was likely the result of two
separate cross linking events which occur at different rates so that the fast event produces
dimers and the slower event covalently bridges these two dimers to produce a tetramer.

If the tetramer is then regarded as a donut with four fold rotational symmetry, then
the octamer may form a double donut with equivalent interfaces in contact with one another.
This double donut type structure is consistent with the observations of Aliev et al. (1986)
based on their electron microscopy imaging of the chick pea enzyme. We can then rationalize
our two-hybrid data in the following way. Monomers assemble into dimers with no two fold
symmetry, but these dimers assemble into tetramers that display four fold symmetry.
Alternatively, there may be no intervening dimer step. Further, all four monomers assemble
in such a way that the tetramer has an "N" face which displays all four amino termini and a
"C" face that displays all four carboxyl termini. To form the octamer, then, two tetramers
assemble with their C faces in contact. This explains why the E310Δ mutant cannot form
octamers; the truncation disturbs the tetramer-tetramer interface.

This model also provides an explanation why our positive two-hybrid transformants
may uniquely reflect the assembly of octamers from tetramers. All the two-hybrid fusions
place the GAL4 domains at the amino terminus. In this arrangement, both the DNA binding
domain and the activation domain are sandwiched between CA and the DNA at the reporter's
promoter. If the overall structure of CA is sufficiently large, it could sterically hinder the
activation domain from interacting with the transcription apparatus (Figure 4.4). Therefore,
our tetramer assembly would recruit the activator domain to the promoter, but it would be
unable to activate transcription. If, however, an octamer formed via a C - C face interaction,
the opposite side of the octamer would display the activator domain as well, and this activator
Figure 4.4 Tentative model of CA's quaternary structure. This model does not necessarily imply that the carboxyl termini must interact with one another.
domain could then interact with the transcription machinery to produce \(\beta\)-galactosidase mRNA.

Obviously, this model is nothing more than speculation, but it is consistent with available data and it does provide a framework in which to test its predictions. First, the model predicts that E310A is a tetramer. This can now be verified using cross linking studies in the same way that other tetrameric CA mutants have been identified (Björkbacka et al., 1997). Secondly, fluorescence spectroscopy can be used to verify that the tetramer has a carboxyl face and an amino face. Fluorescent probes can be attached to these regions and intervening distances can be measured (Liu, 1996). Third, the model predicts that C213A is an unstable octamer. It must be octameric in order to activate transcription in the two-hybrid system, but it must be unstable since the tail can interact with it (Table 4.1). This instability could be verified by equilibrium centrifugation experiments and comparisons could be drawn between the association constants of this mutant and the wild type enzyme.

Overall, we have shown that an element of primary structure at the carboxyl terminus of dicot CAs stabilizes the oligomeric state of the spinach enzyme. Deletion of this extension eliminates the protein's ability to form higher order oligomers. Additionally, we have shown that this domain will interact with the C213A enzyme in the yeast two-hybrid system. The carboxyl tail of the dicot \(\beta\)CAs may therefore be regarded as an important determinant of quaternary structure.

References


CHAPTER 5
CONCLUSIONS

This dissertation has illuminated, for the first time, a structural feature of the β carbonic anhydrases which distinguishes this class from the prototypical α isozymes. A combination of molecular biology and biophysics has been used to show that spinach CA coordinates the active site zinc with two cysteines and one histidine. This is in contrast to the α and γ isozymes both of which bind their respective active site zinc ions with three histidine ligands. Prior to this work, it was generally assumed that the βCA active site would largely mimic the αCA structure, thereby explaining the identical catalytic efficiencies of the two CA classes. The distinction illustrated here may provide a basis to explain the different responses these two classes have to some inhibitors. Furthermore, the active site model proposed here has subsequently been corroborated by an independent lab also using extended X-ray absorption fine structure analysis (Rowlett et al., 1994). This novel ligand scheme now provides additional evidence that the β class is an evolutionarily distinct carbonic anhydrase family. The observation that the metal is bonded to βCA by ligands of very different chemistry than the ligands of the α class may supply fertile ground for exploring how catalytic zinc coordination influences the kinetic characteristics of carbon dioxide hydration in general. In fact, the likelihood for the sulfurs of the cysteine ligands to raise the pKₐ of the catalytic water could prove relevant to the potential regulation of the enzyme's activity by the increase in stromal pH during photosynthesis. Enzymologists will certainly be curious to see if the zinc-sulfur
interactions of βCA impact on the theoretical mechanism of hydroxyl mediated CO$_2$ attack by the enzyme.

It has also been shown that the ten amino acids at the carboxyl terminus of spinach CA have a profound impact on the enzyme’s quaternary structure. Deletion of this element results in an enzyme compromised in its ability to form higher order multimeric complexes. This disruption does not appear to be the result of a secondary disturbance of the enzyme’s tertiary structure as the carboxyl terminus has been shown to independently interact with the full length enzyme. This finding provides a second glimpse at what may be a defining difference between monocots and dicots with respect to carbonic anhydrase, and this difference may impact our understanding of how these two groups of angiosperms differ with respect to carbon fixation in general.

The nature of the interaction between the carboxyl terminus and other CA subunits remains to be determined. However, the model proposed here for multimer architecture generates several testable hypotheses. A detailed investigation of the E310Δ mutant by analytical ultracentrifugation could yield helpful quantitation of the contribution of the carboxy terminal domain to multimer assembly. Further, it may be possible to chemically synthesize the carboxyl terminus for structural analysis using circular dichroism or nuclear magnetic resonance spectroscopy. These approaches might yield a model for how the tail mediates CA assembly. However, such investigations could prove fruitless if the tail requires the presence of other domains in the CA enzyme in order to assume its native conformation.

The locations of the amino and carboxyl termini with respect to one another can also be determined. The model proposed here predicts that amino termini are grouped
together on one face of each tetramer, while the carboxyl termini are grouped on the alternate face, and it is the latter face which forms the tetramer-tetramer interface. If this is so, covalent modification of these termini with appropriate fluorophores can be exploited to measure distances between these regions by fluorescence spectroscopy. The current model would be verified if the labelled amino termini are shown to generate excimers, for example. The same would be true, in turn, for the carboxyl termini.

Overall, it seems clear that the most direct route to understanding the structural features of the beta carbonic anhydrases will be to solve the complete structure of a representative member. Hopefully, an X-ray crystal structure of a βCA will allow investigators to extend our understanding of this enigmatic enzyme.

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Michael Holden Bracey was born on 16 February, 1969, in Shreveport, Louisiana. He grew up and attended school there, graduating from Caddo Parish Magnet High in May, 1987. He moved to Baton Rouge in August, 1987, to attend Louisiana State University with a Chancellor's Alumni Scholarship. Michael received his bachelor of arts degree in May, 1991, in French. Following a year at work as a Research Associate for Dr. Sue G. Bartlett in the Department of Biochemistry, he joined the graduate program at L.S.U. in the fall of 1992. Since then, Michael has worked on carbonic anhydrase, thylakoid protein import, and osmotically induced signal transduction while in Dr. Bartlett's lab. After graduation in May, 1998, Michael plans to join the research group of Dr. Ben Cravatt at the Scripps Research Institute in La Jolla, California, where he will pursue post-doctoral training in neurochemistry and oleamide signaling.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Biochemistry

Title of Dissertation: Structural Characterization of Beta Carbonic Anhydrases from Higher Plants

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