

Carbonic Anhydrase: New Insights for an Ancient Enzyme*[‡]

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Carbonic anhydrase catalyzes the reversible hydration of CO₂
 (Equation 1).

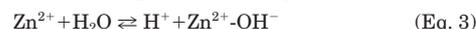
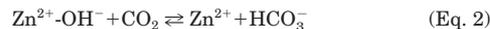


The first carbonic anhydrase was purified from erythrocytes in 1933 (1) followed by the characterization of several mammalian isozymes that dominated research on carbonic anhydrase until recently. Although it has been known since the 1940s that carbonic anhydrase is ubiquitous in plants (2), where it is essential for CO₂ fixation, relatively few studies had been reported. Until 1994, only five carbonic anhydrases had been purified from prokaryotes; however, a recent survey has established that the enzyme is widely distributed among phylogenetically and physiologically diverse prokaryotes, indicating a far greater role for this enzyme in nature than previously recognized (3). The comparison of sequences and crystal structures of the mammalian and plant enzymes demonstrates that they evolved independently and have been designated the α - and β -class, respectively. An additional independently evolved γ -class was reported in 1994 (4) for which phylogenetic analyses predict an ancient origin (3). This review discusses dramatic advances over the past 3 years regarding the structure and biological chemistry of carbonic anhydrases.

Common Properties of the Three Classes

The three classes have no significant sequence identity, and the overall folds (Fig. 1) underscore their independent origins (5). Despite gross structural differences, the active sites of all three classes function with a single zinc atom essential for catalysis (6, 7). Kinetic studies indicate that all three classes employ a two-step isomechanism (8). The first step is the nucleophilic attack of a zinc-bound hydroxide ion on CO₂ (Equation 2). The second step is the regeneration of the active site by ionization of the zinc-bound water molecule and removal of a proton from the active site (Equation 3). In this step, the zinc ion acts as a Lewis acid to lower the

pK_a of the water molecule from ~ 14 to 7.0.



Most carbonic anhydrases have k_{cat} values greater than 10^4 s^{-1} , which requires an intermediate PSR¹ (Equation 4) to transfer the proton from the metal-bound water molecule to the external buffer, "B" (Equation 5).



Proton transport from the active site is the rate-limiting step for enzymes with $k_{\text{cat}} > 10^4 \text{ s}^{-1}$. Thus, k_{cat} is a reflection of the rate of proton transport (Equation 3), whereas the catalytic efficiency (k_{cat}/K_m) is more reflective of the hydration step (Equation 2) and is insensitive to the rate of proton transport. The following sections focus on recent results revealing specific properties of the three classes of carbonic anhydrase, which provide new structural and biochemical perspectives for this enzyme.

The α -Class

General—The α -class is the best characterized with 11 isozymes identified in mammals. Several isozymes are implicated in various disease states for which treatment frequently involves the application of sulfonamides that inhibit carbonic anhydrase activity. Earnhardt *et al.* (9) summarize sulfonamide inhibition constants and maximal k_{cat} and k_{cat}/K_m values for CO₂ hydration by isozymes I–VII. Prokaryotic α -class enzymes are few compared with the other two classes. The recent characterization of mammalian and prokaryotic α -class enzymes has been reported and reviewed (10–15).

Catalysis—The α -class is by far the best studied with respect to the mechanism of catalysis. The reader is referred to excellent reviews of the literature prior to 1999 (6, 7). Recent advancements have focused on the rate-limiting proton transfer step. In several isozymes of the α -class, His-64 accepts a proton from active site water molecules that intervene between the zinc-bound water molecule and His-64. The His-64 PSR can be replaced with other residues that function as PSRs, a result consistent with the proposal that proton transfer occurs through different structures of intervening water chains (16). Indeed, molecular dynamics simulations indicate that the number of intervening waters can vary from two to six (17). Crystal structures of human CA-II show His-64 in either an "in" (toward zinc) or "out" position. This fluctuation of His-64 is postulated to facilitate proton transfer between active site waters and solvent water at the mouth of the active site cavity. Imidazole and imidazole derivatives mimic the PSR function of His-64 and rescue the H64A variant of CA-II that is 10-fold reduced in k_{cat} . Crystal structures of the variant complexed with 4-methylimidazole show the rescue agent occupying the "out" position leading to the conclusion that this orientation of His-64 is important for proton transfer (18). On the other hand, aqueous phase molecular dynamics simulations of the wild-type enzyme in three protonation states indicate that His-64 primarily assumes the "in" orientation, a result leading the authors to suggest that fluctuations between the two orientations of this residue may have limited influence on proton transfer (17). The rate of ¹⁸O exchange between the zinc-bound water molecule and solvent waters is used to determine the rate of intramolecular proton transfer. Fitting Marcus rate theory to the rate data (19) requires a substantial adjustment in large work terms or thermodynamic components suggesting that intramolecular proton transfer involves a reorganization of the active site cavity. It is proposed that the reorganization includes waters not directly involved along the pathway; for example, movement of His-64 from the "out" to the "in" orientation

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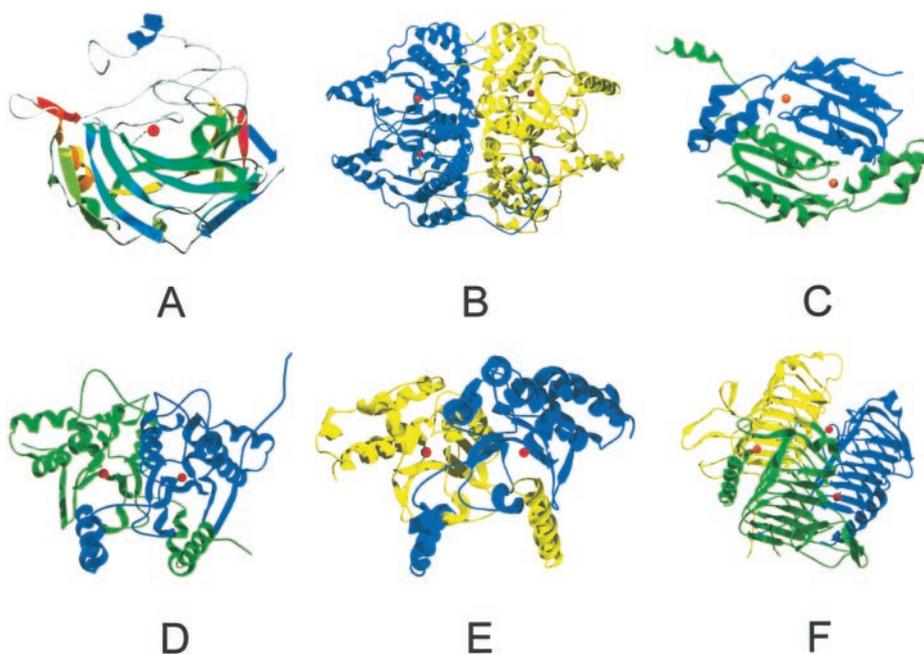
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¹ The abbreviations used are: PSR, proton shuttle residue; MOPS, 2-(N-morpholino) propanesulfonic acid.

FIG. 1. Ribbon diagrams of α -, β -, and γ -class carbonic anhydrase crystal structures. Active site zinc ions are shown as red space-filled spheres; monomer subunits in the β - and γ -class carbonic anhydrases are individually colored. Protein Data Bank structure coordinate files are in parentheses. Molecular masses are for the native enzymes. A, α -class, human isozyme II (4CA2), 30.0 kDa; B, β -class, *P. purpureum* (1DDZ), 62.1 kDa; C, β -class, *P. sativum* (1G5C), dimeric unit of the native octamer, 193.6 kDa; D, β -class, *M. thermoautotrophicum* (1EKJ), dimeric unit of the native tetramer, 75.6 kDa; E, β -class, *E. coli* (116O), dimeric unit of the native tetramer, 98.8 kDa; F, γ -class, *M. thermophila* (1THJ), 69 kDa. Drawings were created using Swiss-PDBViewer software version 3.7b2 available at www.expasy.ch/spdbv/mainpage.html and rendered with Persistence of Vision Ray-Tracer software version 3.1 g available at www.povray.org.



involves breaking H-bonds between the side chain and water. Finally, *ab initio* studies of intramolecular proton transfer indicate that the donor-acceptor distance and the water chain motion are essential to the energetics (20).

Intermolecular proton transfer may involve more than a single PSR (21). Site-specific replacement of Lys-91 and Tyr-131 near the mouth of the active site cavity of isozyme CA-VA produced variant enzymes compromised in k_{cat} but not k_{cat}/K_m , indicating that these basic residues are PSRs. Moreover, kinetic analysis of a double variant suggested a cooperative behavior between the residues in facilitating proton transfer. The incorporation of a histidine analog by chemical modification of the Y131C variant resulted in enhanced proton transfer, a result that further supports the proposed PSR role for Tyr-131 (22).

Metal Affinity—The α -class carbonic anhydrases are characterized by subpicomolar affinities for zinc, which have provided a system for investigating the fundamental properties of metal ion binding in metalloproteins. Recent studies have focused on structural features of the active site and the thermodynamics of solute association that influence metal binding specificity and avidity. The results indicate a role for hydrophobic core residues in human CA-II that are important for preorienting the histidine ligands in a geometry that favors zinc binding and destabilizes geometries that favor other metals (23, 24). Calorimetric studies of CA-II and variants indicate that both desolvation of the metal ion and the binding site have major contributions to the overall thermodynamics, thus directing specificity of binding by optimizing desolvation (25, 26).

The β -Class

General—The understanding of the β -class has lagged far behind that for the α -class; indeed, the first crystal structure for any β -class carbonic anhydrase was reported in 2000 (27). Although initially thought to be composed solely of enzymes from plants, β -class carbonic anhydrases were recently isolated from a variety of algae (28–30) and found to be widely distributed in the Bacteria and Archaea domains (3). The characterization of enzymes from the β -class reveals sharp differences from the other two classes. The α -class and γ -class enzymes are strictly monomers and trimers, respectively; however, members of the β -class are dimers, tetramers, hexamers, and octamers, which suggests a dimer as the basic building block (31). Furthermore, differences in secondary structure are evident from the crystal structures (Fig. 1). Finally, β -class crystal structures reveal that zinc is ligated by two conserved cysteines and one conserved histidine (27, 31, 32).

Diversity—Phylogenetic analyses indicate that the β -class is more diverse than the other two classes (3). Sequence alignment

indicates that only 5 residues, the three zinc ligands plus an aspartate and an arginine, are completely conserved (33). The plant sequences form two monophyletic clades representing dicotyledonous and monocotyledonous plants (3). The remaining sequences are separated into five clades of which one is strongly supported by bootstrapping and appears distantly related to all other clades. This clade, represented by the enzyme “Cab” from the archaeon *Methanobacterium thermoautotrophicum* (32, 33), is composed of sequences primarily from thermophiles in the Archaea and Gram-positive species in the Bacteria. This diversity is supported by recent crystal structures of enzymes from a red alga (*Porphyridium purpureum*, Fig. 1B) and pea (*Pisum sativum*, Fig. 1C), and prokaryotes from the Archaea (*M. thermoautotrophicum*, Fig. 1D) and Bacteria (*Escherichia coli*, Fig. 1E) domains (27, 31, 32, 34). The pea enzyme is a dimer of homodimers whereas the algal enzyme is a homodimer in which the monomer is composed of two internally repeated structures each with an active site. An overlay of the active sites of the *P. sativum* and *M. thermoautotrophicum* (Cab) enzymes shows near perfect alignments of the three zinc ligands and the β -class conserved aspartates and arginines; however, a water molecule is ligated to zinc only in Cab (32). An acetate molecule replaces a water molecule as the fourth zinc ligand in the *P. sativum* enzyme that was crystallized with acetate (31). Surprisingly, the fourth zinc ligand in both the *P. purpureum* and *E. coli* enzymes is the β -class conserved aspartate (27, 34). Residues Gln-151, Phe-179, and Tyr-205 of the *P. sativum* enzyme (31) are conserved among all the eukaryotic and bacterial enzymes in clades A–F; however, they are absent in the sequences of all other carbonic anhydrases that are in the same clade (clade G) as the *M. thermoautotrophicum* enzyme Cab. This observation led Kimber and Pai (31) to propose that the β -class is composed of two subclasses, the “plant type” (represented by the *P. sativum* enzyme) and the “Cab type” (represented by the *M. thermoautotrophicum* enzyme). Both the structural dissimilarities between the two subclasses and their varied responses to inhibitors (10) suggest differences in their mechanism.

Catalysis—Kinetic analyses indicate a zinc hydroxide mechanism for the β -class (33, 35–37). As is the case for the α -class, the zinc-bound acetate in the crystal structure of the *P. sativum* enzyme mimics the binding of bicarbonate in the active site (31). The zinc-bound oxygen of acetate hydrogen bonds with Asp-162 O- δ 1, whereas the second oxygen hydrogen bonds to Gln-151 suggesting a role for these residues in catalysis. The bond between acetate and Asp-162 O- δ 1 is identical to the hydrogen bond between the zinc-bound oxygen of acetate and Thr-199 O- δ 1 of the α -class CA-II isozyme. Thr-199 O- δ 1 functions to orient the zinc-bound hydroxide

for nucleophilic attack on CO₂. Superimposition of active sites also shows that the bond between Gln-151 and acetate in the *P. sativum* enzyme active site overlaps the hydrogen bond between the Thr-199 N and the second oxygen of bicarbonate. Thr-199 N is proposed to electrophilically activate CO₂ by forming a hydrogen bond with CO₂ (6). Therefore, Gln-151 and Asp-162 are thought to play the same roles as Thr-199 of the α -class enzymes (31). Asp-34 in the active site of Cab could function similarly to Asp-162 of the plant-type subclass (32).

The route of proton transfer is not clear from the structures of the *P. sativum* and *P. purpureum* enzymes (27, 31); however, in the structure of Cab, a HEPES buffer molecule is found ~ 8 Å from the zinc (32) and within hydrogen bonding distance of the β -class conserved aspartate (Asp-34), which also forms a hydrogen bond with the zinc-bound water molecule. Thus, one possible pathway for proton transfer is from the zinc-bound water molecule to Asp-34 and then to the sulfate group of HEPES. Indeed, replacement of Asp-34 results in a 10-fold decrease in the k_{cat} of Cab, and the D34A variant is chemically rescued by replacement of MOPS buffer with imidazole at pH 7.2.² These results are consistent with a PSR role for the β -class conserved aspartate in at least the Cab-type subclass.

The structures of the *P. purpureum* and *E. coli* enzymes suggest an additional role for the β -class conserved aspartate, which is a fourth ligand to zinc (27, 34). The presence of a water molecule hydrogen-bonded to the conserved aspartates (Asp-151 and Asp-405) in the duplicated active sites of the *P. purpureum* enzyme leads the authors to propose a modified zinc hydroxide mechanism (27) in which the aspartate functions as a base to abstract a proton from the bound water molecule yielding a nucleophilic hydroxide. The hydroxide moves toward and then binds to the zinc. When bound, the hydroxide attacks CO₂ to generate the zinc-bound bicarbonate. The deprotonated aspartate binds zinc displacing bicarbonate. Finally, to regenerate the active site, a water molecule binds to the aspartate in each of the duplicated active sites. On the other hand, kinetic analysis of variants generated by replacement of the β -class conserved aspartate (Asp-34) in Cab² shows that this residue is not essential for the CO₂ hydration step of catalysis.

The *P. purpureum* and *E. coli* enzymes are only active above neutral pH values, which prompted a second hypothetical model accounting for ligation of the β -class conserved aspartate to zinc. In this model, the aspartate ligand is exchanged with a water molecule above neutral pH values, thereby activating the enzyme (34). A reorganization of residues in the active site coupled to the ligand exchange cannot be ruled out as an additional mechanism for activation; furthermore, it cannot be ruled out that a reorganization of the active site coupled to ligand exchange occurs repeatedly during a single catalytic turnover. Repeated ligand exchange during turnover would potentially allow the conserved aspartate to play a role in proton transfer as established for Cab. Because both the *P. purpureum* and *E. coli* carbonic anhydrases belong to the same phylogenetic clade, other enzymes from this clade may be expected to have a similar active site architecture and mechanism.

The γ -Class

General—The γ -class is thought to have evolved between 3.0 and 4.5 billion years ago (3) and therefore precedes evolution of the α -class at 200–300 million years ago (12, 38). The only γ -class enzyme characterized is “Cam” from the archaeon *Methanosarcina thermophila* (4). Cam is a homotrimer that adopts a left-handed parallel β -helical fold (Fig. 1F) (39). Cam is heterologously produced in *E. coli* at high levels to yield a zinc enzyme (40); however, iron- and cobalt-substituted forms exhibit greater CO₂ hydration rates than the zinc enzyme (41)³; thus, it is possible that Cam functions in *M. thermophila* using a different transition metal than zinc.

Catalysis—Cam employs a metal hydroxide mechanism in catalysis with proton transport as the rate-limiting step (41). Unlike many of the α -class enzymes, Cam does not exhibit esterase activity with *p*-nitrophenyl acetate as the substrate, and the inhibition

by sulfonamides is low compared with the α -class (40). The metal binding site consists of three histidine residues in a tetrahedral geometry similar to that of the monomeric α -class (39); however, in Cam, two of the histidines are donated by one monomer (His-81, His-122) and the other from an adjacent monomer (His-117).

High resolution crystal structures with bicarbonate bound to the active site have led to proposed roles for other active site residues (42), which have been further investigated by site-directed mutational analysis. Solvent-accessible Gln-75 is located with the side chain 5 Å from the zinc and is structurally modeled with the carbonyl oxygen pointed toward the zinc and an amino group hydrogen-bonded to the carbonyl oxygen of the Asn-73 side chain (39, 42). The amino group of Asn-73 is in turn hydrogen-bonded to the side chain hydroxyl group of Ser-114. This hydrogen bond network indicates that the Gln-75 side chain is highly oriented with the carbonyl oxygen forming a hydrogen bond to one of two water molecules coordinated by the zinc (42). Kinetic analyses of the Q75A variant indicate that Gln-75 is important for CO₂ hydration activity.⁴ Thus, Gln-75 may function in analogy with Thr-199 in the α -class CA-II isozyme by hydrogen bonding with and orienting the zinc-bound hydroxide for attack on CO₂.

The carboxylate of Glu-62 resides 5 Å from zinc and has bicarbonate bound in the crystal structure suggesting a potential role in catalysis, although the binding of bicarbonate could be an off pathway event (42). The role for Glu-62 was further investigated by kinetic analyses of variants in which Glu-62 was replaced with several different residues (43). Only the E62D variant maintains wild-type activity whereas several other variants have low k_{cat} and k_{cat}/K_m values suggesting that the negative charge of Glu-62 is important for the CO₂ hydration step in catalysis, although the specific function is unknown.

Glu-84 is adjacent to Glu-62 and assumes two different conformations (42) in analogy with the PSR His-64 in the α -class CA-II isozyme. The replacement of Glu-84 in Cam yields variants with large decreases in k_{cat} relative to wild type but only small changes in k_{cat}/K_m (43). The same variants are rescued up to 46-fold in k_{cat} when assayed in the presence of imidazole, results strongly indicating that Glu-84 functions as a PSR. Interestingly, bicarbonate can function as a proton donor in the dehydration direction of catalysis by Cam and the α -class human CA-II isozyme; however, it is not known if bicarbonate is essential for the proton transfer step in these enzymes (44).

The guanido group of Arg-59 in Cam is located 6 Å from the zinc where it also partners in a salt bridge between Asp-61 and Asp-76 in adjacent monomers (39). The Arg-59 is important for the association of monomers into the native trimer and is essential for the CO₂ hydration step in catalysis.⁵ The specific catalytic function for Arg-59 is unknown; however, it is postulated that this residue may influence the pK_a of the catalytic zinc-bound water molecule or bind bicarbonate as part of the product release cycle.

A δ -Class of Carbonic Anhydrase?

In 1997, Francois Morel and co-workers (45) reported the purification of a 27-kDa monomeric carbonic anhydrase, TWCA1, from the marine diatom *Thalassiosira weissflogii* (45). The catalytic zinc was shown by x-ray absorption spectroscopy to be coordinated by three histidines and a water molecule (46), similar to the active sites of the α -class and γ -class carbonic anhydrases. Additionally, the near-edge spectra argue that the active site geometry is similar to that of α -class enzymes (46). Although no steady-state kinetics have been reported, the existence of a water molecule as a fourth ligand suggests that this enzyme may also follow the zinc hydroxide mechanism of the other three classes. Although TWCA1 has biochemical properties similar to the three known classes of carbonic anhydrase, the deduced sequence of the gene encoding TWCA1 revealed no significant identity to the three classes. In addition, our searches of the sequence data bases failed to identify open reading frames in the Archaea, Bacteria, or Eukarya domains with deduced sequence identity. Thus, TWCA1 is the prototype for a fourth class of carbonic anhydrase that we propose here to be designated the δ -class.

² K. S. Smith, C. Ingram-Smith, and J. G. Ferry, submitted for publication.

³ B. C. Tripp and J. G. Ferry, unpublished results.

⁴ C. Brosius and J. G. Ferry, unpublished results.

⁵ Tripp, B. C., Tu, C., and Ferry, J. G., (2002) *Biochemistry*, in press.

During conditions in which the levels of TWCA1 are low in *T. weissflogii*, a 43-kDa cadmium-specific carbonic anhydrase is expressed (47). The sequence of the gene encoding this carbonic anhydrase has not yet been reported; thus, whether it represents a new class or belongs to a pre-existing class is not yet known.

Conclusions

Undoubtedly, dramatic advances in both the physiology and biochemistry of carbonic anhydrases have been described in the past few years. The catalytic mechanisms for both the β - and γ -class have been further elucidated especially in the proton transfer pathway. It is expected that the recently solved structures of four β -class enzymes will result in significant progress in understanding the mechanism(s) of this class in the near future. Additional enzymes from both the β - and γ -class have been purified and characterized, broadening our knowledge of each class. The report of a fourth class represented by the *T. weissflogii* enzyme suggests we may be only scratching the surface of carbonic anhydrase diversity.

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